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Research report

Thyroid hormone of maternal origin regulates the expression of RC3/neurogranin mRNA in the fetal rat brain

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Abstract

Recent clinical studies indicate that thyroid hormone plays essential roles in fetal brain development. However, the mechanism by which thyroid hormone affects fetal brain development is poorly studied. We recently identified several genes expressed in the fetal cortex whose abundance is affected by thyroid hormone of maternal origin. However, it is unclear whether these genes are directly regulated by thyroid hormone. Because these are the first genes known to be regulated by thyroid hormone during fetal development, we sought to expand our investigation to genes known to be regulated directly by thyroid hormone. We now report that the well-known thyroid hormone-responsive gene RC3/neurogranin is expressed in the fetal brain and is regulated by thyroid hormone of maternal origin. These findings support the concept that maternal thyroid hormone exerts a direct action on the expression of genes in the fetal brain that are important for normal neurological development.

Theme: Development and regeneration

Topic: Hormones and development

Keywords: Thyroid hormone; RC3/neurogranin; Brain development; Cerebral cortex

1. Introduction

Despite the universal acceptance that thyroid hormones are essential for normal brain development [4,15,32,35], the mechanisms by which thyroid hormones produce these effects, the developmental processes they influence, and the timing of their actions are poorly understood. Our lab has recently begun to focus on these issues, with special attention to the timing of thyroid hormone action [13]. This focus is warranted because recent clinical studies demonstrate that thyroid hormone may affect critical features of fetal brain development, and that maternal thyroid dysfunction may result in neurological deficits. For example, children born to pregnant women with untreated hypothyroidism during the second trimester were found to exhibit measurable neurological deficits despite normal circulating thyroid hormone at birth [19,34]. Thus, it is possible that maternal thyroid hormone is essential for normal brain development and may be affecting developmental events during gestation that cannot be repaired after birth.

Although the implications of these clinical findings are important, no experimental work has yet confirmed that thyroid hormone of maternal origin exerts direct effects on the fetal brain. Because receptors for thyroid hormone (TRs) are ligand-activated transcription factors [28–30,32], changes in gene expression are the most direct measure of receptor-mediated effects of thyroid hormone. Moreover, thyroid hormone-induced changes in gene expression in the fetal brain prior to the onset of fetal thyroid function on gestational day (G) 17 [16] must necessarily reflect the action of thyroid hormone of maternal origin. Therefore, we recently initiated a study designed to identify and clone thyroid hormone-responsive genes in the fetal rat brain prior to the onset of fetal thyroid function [14]. We identified several thyroid hormone-responsive genes despite screening only a small proportion of genes expressed in the G16 fetal brain, but none were previously shown to be thyroid hormone-dependent. Therefore, an important remaining question is whether maternal thyroid hormone

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regulates the expression of thyroid hormone-responsive genes in the fetal brain by a direct or an indirect mechanism.

To address this issue, we considered whether known thyroid hormone-responsive genes could be employed as markers of direct thyroid hormone action in the fetal brain. Several genes, including myelin basic protein (MBP) and proteolipid protein 2 (PLP-2), are directly regulated by thyroid hormone during postnatal development [21,40]. However, because these genes are not expressed prenatally in the forebrain [27,41], we clearly could not employ these genes in a study of fetal brain development. In contrast, RC3/neurogranin is a well-characterized thyroid hormone-responsive gene [22–24,31] that may be expressed in the fetal brain [1]. Therefore, we initiated the present study to evaluate whether RC3/neurogranin is expressed in the fetal brain before the onset of fetal thyroid function, and if so, to determine whether its expression is regulated by thyroid hormone of maternal origin.

2. Materials and methods

2.1. 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 expression of RC3/neurogranin throughout normal development, nulliparous female Sprague–Dawley rats ($n=4$; Zivic Miller, Pottersville, PA) were maintained on rat chow and unaltered drinking water ad libitum. The females were paired with males overnight and the presence of sperm in a vaginal smear the following morning indicated mating. This day was defined as G1. Dams were sacrificed by decapitation following CO_2 inhalation at 12.00 h on G14, G16, and G21 and the 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. The remaining dam carried the pregnancy to term. The resulting pups ($n=1$ per postnatal day, P) were sacrificed as described above at 12.00 h on P7, P13, and P19, and the intact head (P7) or brains (P13–P19) were frozen on pulverized dry ice and stored at −80°C. An adult male Sprague–Dawley rat was used to determine the expression pattern in the adult brain.

To test whether thyroid hormone regulates the expression of RC3/neurogranin in the fetal cortex, we evaluated fetal brain tissues derived from the following experiment. Nulliparous female Sprague–Dawley rats ($n=44$; Zivic–Miller) were exposed to the goitrogen 2-mercapto-1-methylimidazole (MMI; Sigma, St Louis, MO; $n=23$) to block the synthesis of thyroid hormone. MMI was dissolved to 0.02% in drinking water and provided fresh daily. Controls ($n=21$) were provided with unaltered drinking water. After 2 weeks of MMI treatment, the females were paired with males as described above. Both hypothyroid (MMI-treated) dams and euthyroid controls (no MMI) were subdivided into five additional groups receiving either no injection or a single subcutaneous (s.c.) injection of thyroxine (T_4; 12.5 μg/kg body weight [BW]; Sigma) at either 09.00 h or 21.00 h on either G14 or G15. These injections were therefore timed to occur 48, 36, 24, or 12 h before sacrifice at 09.00 h on G16. At 09.00 h on G16, all dams were sacrificed and fetuses were collected and stored at −80°C as described above. Radioimmunoassay (RIA) of total T_4 and thyrotropin (TSH) in serum of the dams confirmed the efficacy of our treatment [14].

2.2. 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 the G14 fetus and one G16 fetus per dam and coronal sections were collected from the remaining brains of animals in the developmental study. Sections were thaw-mounted onto gelatin-coated microscope slides and stored at −80°C until hybridization. In situ hybridization of the RC3/neurogranin probe was performed as described previously [39], with several exceptions. First, the sections were immersed for 30 min in 4% formalin. Second, the hybridization buffer did not contain single-stranded DNA, but did contain 0.1% sodium pyrophosphate and 200 mM dithiothreitol rather than 50 mM. Third, the hybridization was performed at 52°C. Fourth, after the hybridization, the ribonuclease treatment was performed in a buffer containing 10 mM Tris/1 mM EDTA/2×SSC, pH 7.4.

2.3. Probes

The pRc/CMV cDNA containing RC3/neurogranin was kindly provided by Dr Juan Bernal (Madrid, Spain). Both complementary and sense-strand RC3/neurogranin RNA probes were generated in vitro in the presence of 1 μg linearized plasmid, 500 μM each of GTP, ATP, and CTP and 12 μM of UTP (UTP+^{33}P-UTP at a molar ratio of 1:1). The RC3/neurogranin cDNA was linearized with *Hind*III and transcribed in the presence of Sp6 polymerase for complementary RNA synthesis; it was linearized with *Apa*I and transcribed in the presence of T7 polymerase for sense-strand RNA synthesis. In each case, the DNA template was removed by DNAse digestion and the RNA probe was purified by phenol–chloroform extraction followed by two ethanol precipitations.

2.4. Autoradiography and signal quantitation

Following in situ hybridization, all slides were arranged in X-ray cassettes and apposed to BioMax film (Kodak,

Rochester, NY) for either 2 weeks (hybridizations on prenatal tissue) or 16 h (hybridizations on postnatal tissue). ^{14}C-standards (American Radiolabeled Chemicals, Inc., St Louis, MO) were simultaneously apposed to the film to verify that the film was not over-exposed. Hybridization signal was analyzed as described previously [39] 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. The relative abundance of RC3/neurogranin mRNA was measured over the cortex of G16 fetal brains using the thresholding function in which all pixels containing density values exceeding a minimum value were averaged over the specified brain area. The resulting values were averaged over four sections for each fetus, with one fetus per litter and 4 to 6 litters per treatment group.

2.5. Statistical analysis

Outliers, defined as those values exceeding 1.5 interquartile ranges from the upper and lower quartiles, were eliminated using a box and whisker plot (Statistix, Analytical Software, Tallahassee, FL). A two-way analysis of variance (ANOVA) was performed on imaging data using the Statview statistical package (Abacus Concepts, Berkeley, CA), followed by *t*-tests between individual means.

3. Results

RC3/neurogranin expression was observed at the earliest time-point examined (G14) and exhibited widespread expression throughout development (Fig. 1). Specificity of the hybridization was evaluated using a sense-control probe of equivalent specific activity. Specific RC3/neurogranin signal was restricted to the nervous system at early developmental stages as has been shown previously for the neonate and adult [1]. RC3/neurogranin expression was robust in the hypothalamus on G16 as it was in the ventromedial hypothalamus (VMH) in later developmental stages (Fig. 1 and [1]). In the cortex, RC3/neurogranin mRNA was expressed in both the intermediate and ventricular zones on G16 (nomenclature derived from [6]), a developmental time characterized by intense proliferation and migration of cortical neurons [9]. As development proceeds, RC3/neurogranin in the cortex becomes preferentially expressed in layers IV–II. The hippocampus exhibited intense RC3/neurogranin expression throughout development. Generally, the abundance of RC3/neurogranin mRNA increased until approximately P19; expression in the adult brain was considerably lower in abundance than at this pre-weaning period.

Quantitative analysis of film autoradiograms produced by manipulating maternal thyroid status on G16 revealed that fetuses derived from hypothyroid dams (MMI-treated) exhibited significantly lower levels of RC3/neurogranin than fetuses derived from euthyroid dams (Fig. 2). This overall reduction was reflected by a significant F for the main effect of MMI treatment ($F_{(1,28)}=5.163$, $P<0.04$) in the absence of a significant F for the main effect of T_4 or a significant interaction. Moreover, post-hoc analysis of RC3/neurogranin expression in hypothyroid versus control fetuses given no injection revealed a significant difference between means ($P<0.05$, Fig. 2).

4. Discussion

The present study demonstrates for the first time that thyroid hormone of maternal origin can selectively regulate the expression of a well-characterized thyroid hormone-responsive gene in the fetal brain. Clinical studies find that thyroid hormone of maternal origin may be important in fetal brain development [8,19,34]. However, few experimental studies have focused on clarifying whether or how thyroid hormone affects brain development during fetal life. It is undisputed that TRs are expressed in the fetal brain of humans [5] and rat [7]. But some investigators argue that TRs are inhibited from responding to thyroid hormone until after birth [2,32,38]. Therefore, the present study is important because it demonstrates clearly that thyroid hormone of maternal origin is capable of affecting the expression of a known thyroid hormone-responsive gene in the fetal brain.

It is important to recognize that the finding that RC3/Neurogranin expression is regulated by thyroid hormone in the adult brain [24] does not imply that it is regulated by thyroid hormone at all developmental times. Our reasoning is that RC3/Neurogranin expression is not regulated by thyroid hormone in all neurons that contain TRs [22]. Therefore, the responsiveness of RC3/Neurogranin expression to thyroid hormone is itself regulated in some manner that is not completely understood. This observation also implies that responsiveness to thyroid hormone is not solely a function of the gene itself, but rather is a function of the gene, the specific cell type, and the developmental stage. Schwartz et al. [38] demonstrate clearly for the gene PCP-2 that there is a developmental period during which it is sensitive to thyroid hormone regulation. Moreover, Anderson et al. [2] suggest that the transcription factor COUP-TF may play the role of blocking premature regulation by thyroid hormone of PCP-2. Observations similar to these on the pleiotropic nature of thyroid hormone action are well-documented [20,25,28,29]. Thus, our present data represent an important component of the evidence indicating that thyroid hormone of maternal origin can directly regulate the expression of specific genes in the fetal brain.

Untreated congenital hypothyroidism is known to have profound effects on neurological development in children

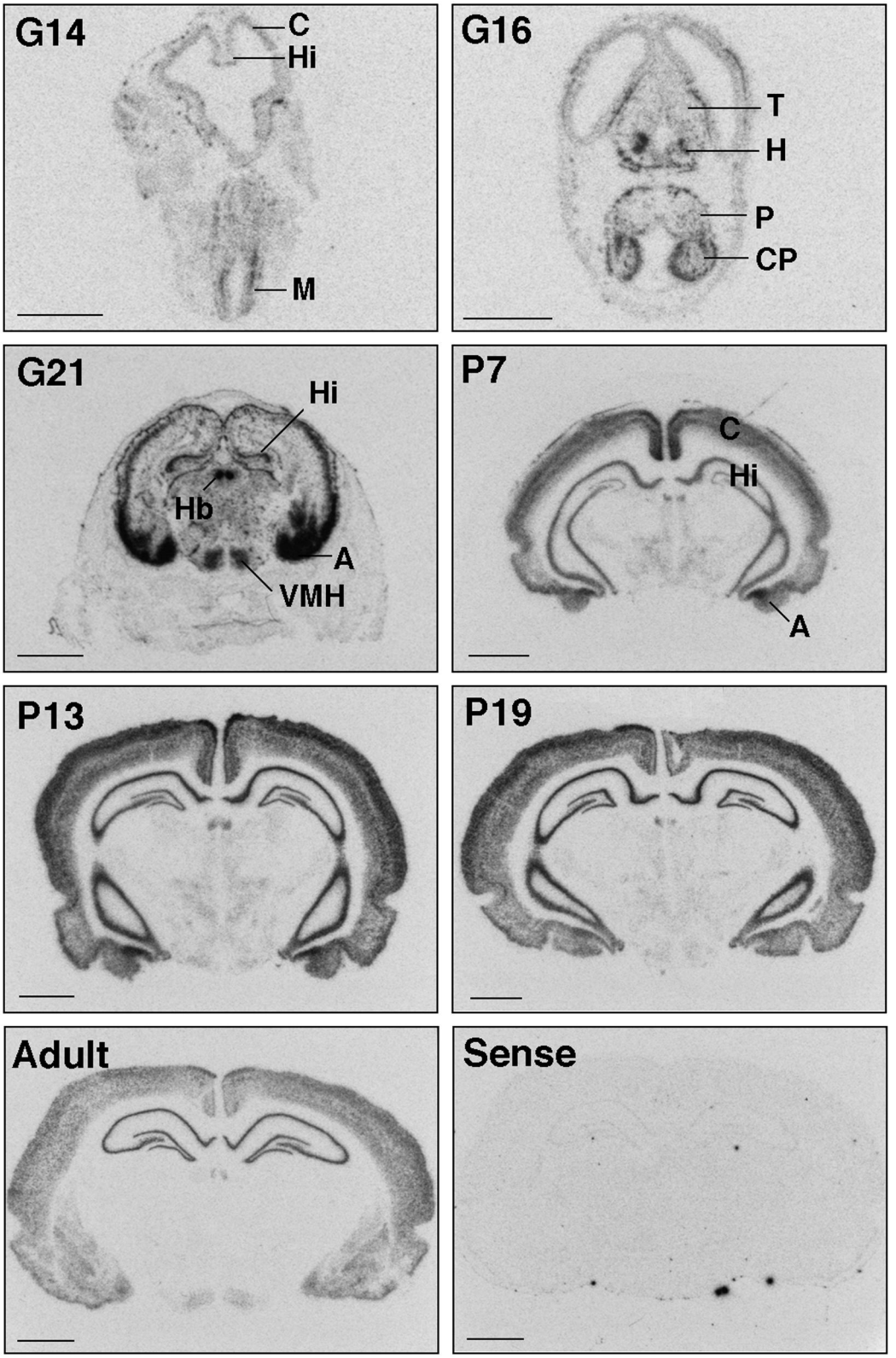

Fig. 1. Distribution of RC3/neurogranin mRNA during development in the euthyroid rat. Images are derived from film autoradiograms following in situ hybridization and apposition to film for either 2 weeks (G14, G16, G21, sense) or 16 h (P7, P13, P19, adult). RC3/neurogranin probes were applied to coronal sections of G14 embryo, G16 embryo, G21 head, P7 head, P13 brain, P19 brain, or adult brain. Sense controls of embryonic tissue contain the edge artifact seen in the first three panels. All other signal is above background. A, amygdala; C, cortex; CP, cerebellar primordium; H, hypothalamus; Hb, habenula; Hi, hippocampus; P, pons; T, thalamus; VMH, ventromedial nucleus of the hypothalamus. Scale bar=0.2 cm.

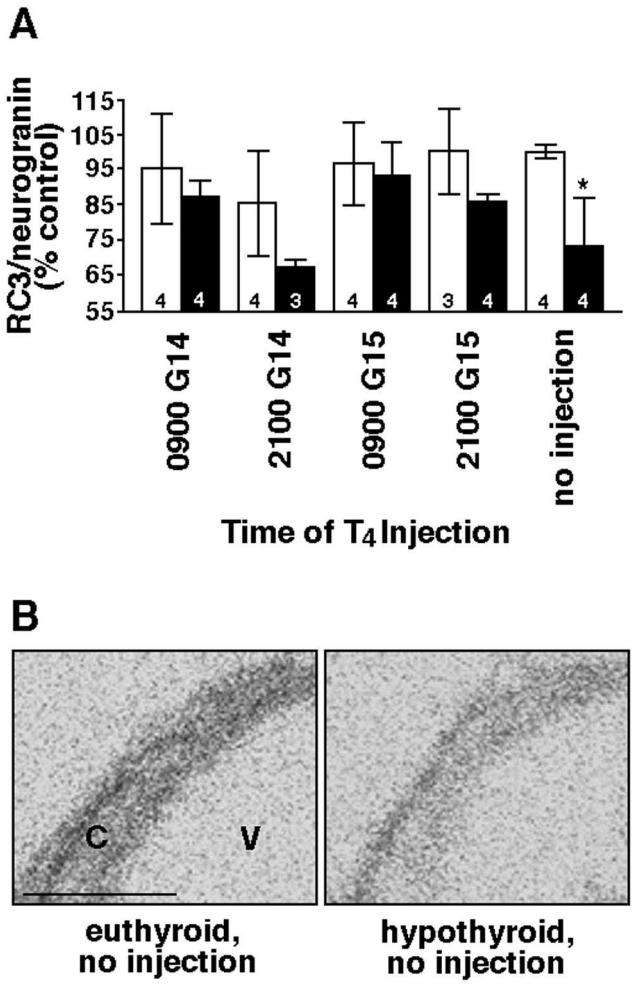

Fig. 2. Effect of chronic MMI treatment and acute T_4 injections on RC3/neurogranin expression in the G16 fetus. (A) Quantitative analysis of film autoradiograms following in situ hybridization for RC3/neurogranin are described in Materials and methods. Bars represent mean±S.E.M. of the film density (converted to % controls ±CV) over the cortex, with number of dams per group noted within each bar. All animals were sacrificed at 09.00 h on G16. Outliers were eliminated as described in Materials and methods. Groups differed in the timing of T_4 injection as shown below the ordinate. Open bars, euthyroid dams (no MMI); closed bars, hypothyroid dams (MMI). (B) Film autoradiograms following in situ hybridization of RC3/neurogranin, illustrating the difference in signal intensity between control animals and those treated with MMI, depicted graphically in A. C, cortex; V, lateral ventricle. Scale bar=0.5 mm. *significantly different from euthyroid controls ($P<0.05$).

[26]. Neonatal screening for T_4 and subsequent T_4 supplementation has greatly diminished these consequences [17,42]. However, it is becoming clear that maternal hypothyroidism also can have devastating effects on neurological development even if the neonate is not hypothyroid at birth. For example, cretinism is a particularly severe consequence of maternal hypothyroidism that occurs in geographical areas in which dietary iodine is very low [11]. However, neurological cretinism can be prevented by iodine supplementation only if initiated during pregnancy [8,12], suggesting that thyroid hormone of maternal origin plays a role in fetal brain development. In addition, infants born to women shown to be hypothyroid at 12 weeks gestation performed significantly more poorly on neurological tests at 10 months of age compared to infants born to women with normal T_4 levels [34]. Moreover, children born to women with undiagnosed hypothyroidism during the second trimester exhibited neurological deficits when tested at 7–9 years old despite being euthyroid from birth [19]. Taken together, these data strongly suggest that thyroid hormone of maternal origin is essential for normal neurological development.

It will be important to identify specific neurodevelopmental processes regulated by maternal thyroid hormone and to delineate the molecular mechanisms underlying this regulation and the functional consequences of disrupted thyroid hormone action during fetal development. The functional characteristics of RC3/neurogranin may provide some insight into these issues. Specifically, RC3/neurogranin is a protein kinase C (PKC) substrate that binds calmodulin in the absence of Ca^{2+} [3,18,36]. Phosphorylation of RC3/neurogranin by PKC results in its dissociation from calmodulin during long-term potentiation (LTP) [10], suggesting that the protein plays a role in synaptic plasticity. This role has recently been supported by studies in mice with a targeted deletion of the gene encoding PKCγ. These animals exhibit spatial learning deficits and impaired hippocampal LTP, possibly because RC3/neurogranin is not phosphorylated in these mice [37]. Considering that thyroid hormone levels affect spatial learning in the offspring [33], it is possible that RC3/neurogranin mediates some of the effects of maternal hypothyroidism on fetal brain development.

The present data are consistent with our previous report that some thyroid hormone-responsive genes in the fetal cortex retain their sensitivity to thyroid hormone to adulthood [14]. Specifically, RC3/neurogranin expression is known to be regulated by thyroid hormone in the neonate and adult [22–24,31], and the present findings demonstrate that it is thyroid hormone-responsive in the fetus. Likewise, NSP and Oct-1 expression are sensitive to thyroid hormone in the fetus and in the adult [14]. Thus, we propose that the 'critical period' of thyroid hormone sensitivity of the developing brain depends on the brain region and developmental process under consideration, as well as the specific gene being evaluated. Clearly, the brain growth-spurt that occurs shortly after birth in the rat is highly sensitive to thyroid hormone [32]. However, our present and previous findings clearly indicate that this is not a unique period of thyroid hormone sensitivity.

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