Thyroid Hormone Regulates reelin and dab1 Expression During Brain Development

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The reelin and dab1 genes are necessary for appropriate neuronal migration and lamination during brain development. Since these processes are controlled by thyroid hormone, we studied the effect of thyroid hormone deprivation and administration on the expression of reelin and dab1. As shown by Northern analysis, in situ hybridization, and immunohistochemistry studies, hypothyroid rats expressed decreased levels of reelin RNA and protein during the perinatal period [embryonic day 18 (E18) and postnatal day 0 (P0)]. The effect was evident in Cajal-Retzius cells of cortex layer I, as well as in layers V/VI, hippocampus, and granular neurons of the cerebellum. At later ages, however, Reelin was more abundant in the cortex, hippocampus, and olfactory bulb of hypothyroid rats (P5), and no differences were detected at P15. Conversely, Dab1 levels were higher at P0, and lower at P5 in hypothyroid animals.

In line with these results, reelin RNA and protein levels were higher in cultured hippocampal slices from P0 control rats compared to those from hypothyroid animals. Significantly, thyroid-dependent regulation of reelin and dab1 was confirmed in vivo and in vitro by hormone treatment of hypothyroid rats and organotypic cultures, respectively. In both cases, thyroid hormone led to an increase in reelin expression. Our data suggest that the effects of thyroid hormone on neuronal migration may be in part mediated through the control of reelin and dab1 expression during brain ontogenesis.

Key words: reelin, dab1, thyroid hormone, neuronal migration, cortical lamination, brain development

Neuronal migration is an essential step in the genesis of the nervous system, particularly in laminated brain regions (Marín-Padilla, 1971, 1998; Rakic, 1988, 1990; Hatten, 1993). Abnormal migration of neurons has been linked to cognitive deficits, mental retardation, and motor disorders (Eksloglu et al., 1996; Sheldon and Padilla, 1971, 1998; Rakic, 1980, 1992; Derer, 1985; Rakic and Caviness, 1995). Characterization of the defective gene in these mice showed that it encoded a large extracellular protein, Reelin, that is expressed in discrete regions of the developing brain (D’Arcangelo et al., 1995, 1997; Hirotsune et al., 1995; Ogawa et al., 1995). reelin is expressed by different sets of neurons, including the pioneer Cajal-Retzius (CR) cells in layer I of the cerebral cortex and the granule cells of the cerebellum (D’Arcangelo et al., 1995; Ogawa et al., 1995; Miyata et al., 1996; Nakajima et al., 1997; Schiffman et al., 1997; Alcántara et al., 1998). In addition to controlling neuronal position, Reelin influences the growth and targeting of hippocampal afferents (Del Río et al., 1997; Borrell et al., 1999).

The study of mouse mutants has led to identify some of the molecules that regulate neuronal positioning. The reeler mutant has severe abnormalities in the neocortex, hippocampus, and cerebellum (Caviness and Sidman, 1973; Mariani et al., 1977; Goффinet, 1980, 1992; Derer, 1985; Rakic and Caviness, 1995). Characterization of the defective gene in these mice showed that it encoded a large extracellular protein, Reelin, that is expressed in discrete regions of the developing brain (D’Arcangelo et al., 1995, 1997; Hirotsune et al., 1995; Ogawa et al., 1995). reelin is...
causes an array of abnormalities in the CNS of which alterations of cell migrations are of special relevance. In rodents, there is a delayed migration of cerebellar granule neurons, positional alterations of Purkinje cells, and abnormalities in cerebral cortex lamination with ectopic location of neurons (Patel et al., 1976; Legrand, 1984; Berbel et al., 1993; Lucio et al., 1997).

A number of brain genes have been identified as regulated by thyroid hormone. They include those coding for the major myelin proteins, cytoskeletal proteins, neurotrophins and their receptors, transcription factors, and intracellular signaling proteins (for review, see Oppenheimer and Schwartz, 1997; Bernal and Guadán-Ferraz, 1998). None of these target genes are directly involved in neuronal migration. Therefore, we investigated whether the expression of two genes critical for neuronal migration, reelin and dab1, is regulated by thyroid hormone. We show that reelin expression is severely decreased in hypothyroid rats at the perinatal stage, but dab1 mRNA expression is not altered. Interestingly, Dab1 accumulates whereas the level of Reelin is reduced. Significantly, hormone treatment restores the normal pattern of reelin expression in vivo and in vitro, indicating that the hormone is involved in the regulation of the reelin gene.

MATERIALS AND METHODS

Animals. Wistar rats maintained in the animal facilities of our Instituto de Investigaciones Biomedicas were used for the studies reported here. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to use alternatives to in vivo techniques. The maintenance and handling of the animals were as recommended by the European Communities Council Directive of November 24th, 1986 (86/609/EEC). To induce fetal and neonatal hypothyroidism (E18, P0, P5, P15), animals at embryonic day 18 (E18) and postnatal day 0, P5, and P15 were treated with T3 for 24 hr after birth. T4 was used for the in vivo, in vitro, and in situ experiments. T3 treatment was obtained by cutting tissue pieces in a McIlwain tissue chopper (Mickle Technologies, Gaithersburg, MD), mounted on gelatin-coated slides and covered with Mowiol. Immunocytochemistry, embryos and postnatal rats were perfused with 2% paraformaldehyde and sectioned as above. Sections were incubated with the CR50 monoclonal antibody (dilution 1:2000) that recognizes the N-terminal region of Reelin (Ogawa et al., 1995; D’Arcangelo et al., 1997). The primary antibody was visualized using a biotinylated secondary antibody (1:200) and a streptavidin–peroxidase complex (1:400) (Vector Laboratories, Burlingame, CA). Peroxidase reactions were developed using diaminobenzidine (DAB) and H2O2. For reelin antibody, we used a rabbit polyclonal antibody (B3; dilution 1:2000) previously described (Howell et al., 1997a).

Organotypic slice cultures. Hippocampal slice cultures were prepared from normal (n = 10) and hypothyroid rats (n = 10) essentially as described (Del Rio et al., 1996, 1997). P0 animals were anesthetized by hypothermia, and the hippocampus and the prospective parietal cortex were dissected out. Transverse slices (300-350-mm-thick) were obtained by cutting tissue pieces in a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, UK). Selected slices were maintained in Minimum Essential Medium (MEM) supplemented with l-glutamine (2 mM) for 45 min at 4°C. Thereafter, slices were cultured using the membrane interphase technique (Stoppini et al., 1991). Incubation medium was 50% MEM, 25% horse serum, 25% HBSS, supplemented with l-glutamine (2 mM). Experimental groups comprised cultures established from hypothyroid pups incubated in normal serum (n = 24) or in T3/T4-depleted serum (n = 24) with or without daily added T3 (150 nM, n = 24, or 500 nM, n = 24). Organotypic cultures from newborn control rats were distributed in similar groups: normal serum (n = 24), T3/T4-depleted serum alone (n = 24), or supplemented daily with T3 (150 nM, n = 24, or 500 nM, n = 24). After 6 days in vitro, representative cultures were fixed with 4% paraformaldehyde in 0.1 mM phosphate buffer and stored. After several rinses, 50-μm-thick sections were obtained using a vibratome, and processed for the detection of reelin mRNA and protein by in situ hybridization and immunocytochemistry as described above.

For quantitative RT-PCR analysis, total RNA from six organotypic slices was extracted by non-denaturing methods (Sambrook et al., 1989) and resuspended in 20 μl of distilled water. Four microliter aliquots were retrotranscribed and amplified by using Retrotools cDNA/DNA polymerase kit (Biotools, Madrid, Spain) according to the manufacturer’s instructions (labeling was performed at Tm – 3°C, and amplification was run up to 25 cycles, with Taq polymerase). For reelin assays, the forward primer was ATACGTGAGATCATCGTCTTATCATTCTCGTGGTG, and the reverse primer was ATACGTGAGATCATCGTCTTATCATTCTCGTGGTG, and the reverse primer was GCCCTCCCTTGGAAAGCTGTTG, and the reverse primer was CTTGGGAGCCATGTAGGCG, covering a 435 bp coding sequence between nucleotides 608 and 1043 of mouse GAPDH cDNA (Sabath et al., 1990). PCR products were run in 1.2% agarose gels, then transferred to nylon membranes, and probed with a mouse 3primed forward primer by standard procedures (Sambrook et al., 1989). Results were analyzed in an Instant Imager apparatus (Packard, Meridian, CT), and data were expressed as counts per minute.

Brain extract preparation and immunoblot analysis. Protein extracts

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from the cerebral cortex and cerebellum of P0 and P5 control and hypothyroid rats, and total brains from E17 or newborn wild-type and reeler mutant mice were prepared by dounce homogenizing the tissue that was snap-frozen in liquid nitrogen in 500 μl of ice-cold lysis buffer (0.1% NP-40, 250 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM PMSF, 20 μg leupeptin, and 50 mM NaF) per 100 mg tissue. Extracts were cleared by centrifugation at 14,000 rpm for 30 min. One hundred micrograms of protein extract was loaded per lane onto a 4–12% polyacrylamide gradient gel (Novagen, Madison, WI), electrotransfered to nitrocellulose membranes, incubated with a rabbit polyclonal antibody directed against the phosphotyrosine binding (PTB) domain of mDabl (antibody PTB31) and visualized by enhanced chemiluminescence (Boehringer Mannheim). Blots were stripped and reprobed with an antibody PTB31 and visualized by enhanced chemiluminescence (Boehringer Mannheim). Controls. Control hybridizations with sense digoxigenin-labeled riboprobes, or RNase A digestion before hybridization, prevented alkali-labile phosphatase staining above background levels. In immunocytochemical controls, omission of the primary antibody prevented dianinobenzidine staining. For RT-PCR assays, RNA samples were digested with DNase I by standard procedures (Sambrook et al., 1989) and then subjected to direct PCR analysis.

Controls. RNA expression was decreased in layer I/SLM than at previous stages, although a substantial number of positive neurons was still present in layer I/SLM (Fig. 2F). Both the number of positive neurons and their intensity of labeling were lower in layer I/SLM than at previous stages, although a substantial number of positive neurons was still present in layer I/SLM (Fig. 2F). No significant differences were found in these layers in the number of positive neurons between control and hypothyroid rats (Fig. 3), although neurons displayed weaker signals in hypothyroid rats (Fig. 2F). In contrast, the number of reelin-positive neurons in layers II-VI was decreased in hypothyroid rats (Fig. 3).

At P5 the levels of reelin RNA expression were slightly lower in layer I/SLM than at previous stages, although a substantial number of positive neurons was still present, particularly in the hippocampus (Fig. 2CJ). No significant differences were found in these layers in the number of positive neurons between control and hypothyroid rats (Fig. 3), although neurons displayed weaker signals in hypothyroid rats (Fig. 2F). In contrast, the number of reelin-positive neurons in layers II-VI was decreased in hypothyroid rats (Fig. 3).

At P15, reelin RNAs were detected in a few neurons in layer I of the neocortex and in neurons scattered within layers II-VI (Fig. 2E), which are known to correspond to certain GABAergic neurons (Alcántara et al., 1998). In the hippocampus, reelin RNA expression was still detected in CR cells as well as in some interneurons distributed within the plexiform layers (Fig. 2K). The pattern of expression in hypothyroid rats was similar to that in controls (Figs. 2F, 3). These data demonstrate that reelin RNA expression is downregulated by hypothyroidism at late prenatal and early postnatal stages of corticogenesis.

Distribution of Reelin immunoreactivity in the cerebral cortex of hypothyroid rats

To investigate whether Reelin distribution was also altered in hypothyroid rats, brain sections were immunostained with the CR50 mAb that recognizes the N-terminal region of Reelin (Ogawa et al., 1995; D’Arcangelo et al., 1997). At E18–P0, CR50 immunoreactivity was very prominent in neurons present in the marginal zone of the neocortical and hippocampal anlage [prospective layer I and stratum lacunosum-moleculare (SLM), respectively] (Figs. 2A,G, 3). These neurons were intensely labeled and displayed large, horizontally-oriented perikarya, which are typical for CR cells (Soriano et al., 1994; Del Río et al., 1995, 1997; Alcántara et al., 1998). In agreement with earlier studies (Schiffman et al., 1997; Alcántara et al., 1998), reelin-positive CR cells were more numerous in the hippocampus than in the neocortex (data not shown). The pattern of reelin expression at E18 and P0 was similar in layer I/SLM. A second population of reelin-positive neurons was detected at P0 in layers V/VI of the neocortex (Fig. 2A) and in the plexiform layers of the hippocampus (Schiffman et al., 1997; Alcántara et al., 1998).

In hypothyroid rats, the regional and laminar patterns of reelin expression at E18–P0 were similar to those in control rats. However, the number of labeled neurons in layer I of the neocortex and in the SLM of the hippocampus was significantly lower, particularly at P0 (Figs. 2B, 3). Moreover, reelin-positive CR neurons clearly displayed weaker hybridization signals in hypothyroid rats than in controls (Fig. 2H). Both the number of positive neurons and their intensity of labeling were lower in layers V/VI of the neocortex and in the hippocampal plexiform layers at P0 (Figs. 2B, 3).

Distribution of Reelin immunoreactivity in the cerebral cortex of hypothyroid rats

To determine whether reelin expression was altered by thyroid hormone depletion in the developing rat brain, we first analyzed reelin mRNA levels by Northern blot hybridization. As shown in Figure 1, reelin RNA expression was markedly downregulated in the cerebral cortex of hypothyroid rats at P0 (50–60% decrease). Recent studies have shown that reelin is differentially expressed in several regions of the developing brain (Schiffman et al., 1997; Alcántara et al., 1998; Rice et al., 1998). To gain insight into the regional differences in the regulation of reelin expression by thyroid hormone, we performed in situ hybridization analyses. We first focused on the pattern of developmental expression in the neocortex and hippocampus, two regions that are targets of thyroid hormone action. At E18–P0 reelin transcripts were very prominent in neurons present in the marginal zone of the neocortical and hippocampal anlage [prospective layer I and stratum lacunosum-moleculare (SLM), respectively] (Figs. 2A,G, 3). The pattern of reelin expression at E18 and P0 was similar in layer I/SLM. A second population of reelin-positive neurons was detected at P0 in layers V/VI of the neocortex (Fig. 2A) and in the plexiform layers of the hippocampus (Schiffman et al., 1997; Alcántara et al., 1998).

The pattern of reelin expression at E18 and P0 was similar in layer I/SLM. A second population of reelin-positive neurons was detected at P0 in layers V/VI of the neocortex (Fig. 2A) and in the plexiform layers of the hippocampus (Schiffman et al., 1997; Alcántara et al., 1998).

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At P5 the levels of reelin RNA expression were slightly lower in layer I/SLM than at previous stages, although a substantial number of positive neurons was still present, particularly in the hippocampus (Fig. 2CJ). No significant differences were found in these layers in the number of positive neurons between control and hypothyroid rats (Fig. 3), although neurons displayed weaker signals in hypothyroid rats (Fig. 2F). In contrast, the number of reelin-positive neurons in layers II-VI was decreased in hypothyroid rats (Fig. 3).

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Figure 2. Effects of hypothyroidism on reelin RNA expression in the cerebral cortex. A–F, Pattern of reelin RNA expression in the neocortex of control (A, C, E) and hypothyroid (B, D, F) rats at P0 (A, B), P5 (C, D), and P15 (E, F). Cortical layers are indicated to the right. Note the decreased RNA levels in hypothyroid rats at P0 and P5. Arrowheads in A and B point to CR cells. G, H, High magnification photomicrographs illustrating reelin RNA-positive CR cells in layer I of the neocortex in control (G) and hypothyroid rats (H). I, J, Distribution of reelin RNA-positive cells in the hippocampus of control (I) and hypothyroid (J) rats at P5, showing decreased RNA levels both in the stratum lacunosum-moleculare and in the remaining hippocampal layers. K, Distribution of reelin RNA-positive cells in the hippocampus of a control rat at P15. No differences were detected in hypothyroid rats at this age in this region. C, Control; H, hypothyroid; I–VI, cortical layers; CP, cortical plate; DG, dentate gyrus; GL, granule cell layer; ML, molecular layer; CA3, CA1, hippocampal subdivisions CA3 and CA1; SLM, stratum lacunosum-moleculare. Scale bars: A, 200 μm (applies to B–D, I, J); E, 100 μm (applies to F, K); G, 40 μm (applies to H).
Immunoreactive neurons were difficult to identify in layer I/SLM of newborn hypothyroid rats, and the diffuse staining observed in control rats was drastically reduced (Fig. 4B,F). Furthermore, a few Reelin-positive neurons were observed in layers V-VI of the cortex and in the hippocampal plexiform layers in control rats, which could not be detected in hypothyroid rats (data not shown).

At P5, the pattern of Reelin immunostaining in layer I/SLM of control rats was similar to that seen at previous stages (Fig. 4C,G). In contrast to the perinatal stages, a large number of CR50-positive neurons were also present in layer I/SLM of P5 hypothyroid rats, with the diffuse extracellular-like staining being very prominent (Fig. 4D,H). In fact, the intensity of immunolabeling at P5 was slightly higher in hypothyroid rats than in control rats. At P15 a few Reelin-positive neurons populated layer I/SLM and the remaining neocortical and hippocampal layers in control rats. No clear differences were apparent in the distribution of Reelin-positive neurons in hypothyroid rats (Fig. 4I-L), although cells were more weakly stained in these experimental animals. Taken together, the data show that Reelin levels are decreased at perinatal stages in hypothyroid rats, whereas they appear to reach normal levels at later postnatal stages.

**reelin expression in the cerebellum and olfactory bulb of hypothyroid rats**

We next examined the developmental distribution of reelin mRNA and protein in the cerebellum and olfactory bulb, two
Figure 4. Reelin expression in the cerebral cortex of control and hypothyroid rats. A–D, Photomicrographs showing the distribution of CR50 immunostaining in layer I of control (A, C) and hypothyroid rats (B, D) at P0 (A, B) and P5 (C, D). Some CR50-positive CR cells are indicated by arrowheads. Note the decreased staining at P0 in hypothyroid animals. E–H, Pattern of CR50 immunostaining in the hippocampus of control (E, G) and hypothyroid rats (F, H) at P0 (E, F) and P5 (G, H), illustrating a clear reduction in the staining in hypothyroid rats at P0. The hippocampal fissure is indicated by arrowheads. I–L, Pattern of CR50 staining in the neocortex (I, J) and hippocampus (K, L) of control (I, K) and hypothyroid (J, L) rats at P15. No marked differences were found in both cortical regions at this age. C, Control; H, hypothyroid; EC, entorhinal cortex; other abbreviations are as in legend to Figure 2. Scale bars: A, 40 μm (applies to B–D); E, 200 μm (applies to F); G, 200 μm (applies to H); I, 100 μm (applies to J–L).
regions of high reelin expression in which migration deficits have been reported in hypothyroid rats (Patel et al., 1976; Legrand, 1984). At E18–P0 reelin transcripts and CR50 immunoreactivity in the cerebellum were detected in the external granule cell layer (EGL) and in a population of neurons in the prospective internal granule cell layer (IGL), which may correspond to the first postmigratory granule cells (see Miyata et al., 1996). Both the hybridization and the immunocytochemical signals were lower in the cerebellum of hypothyroid rats (Fig. 5A,B). In contrast, no remarkable changes in expression levels or immunohistochemical signals were observed in the primordium of the olfactory bulb at these ages (data not shown).

At P5–P15 reelin mRNA and CR50 immunolabeling were prominent in both the EGL and the IGL of the cerebellum (Fig. 5C,E,G,I). Although the EGL is thicker in the hypothyroid rat brain because of the delayed migration of granule cells, the distribution of reelin RNA and protein was similar in control and hypothyroid rats at P5–P15 (Fig. 5D,F,H,J). However, both mRNA and protein levels were clearly elevated in the cerebellum of hypothyroid rats at these ages. In the olfactory bulb, decreased levels of RNA and protein were noticed at P5 in hypothyroid rats (Fig. 5K,L), whereas no changes were detected at P15.

**Developmental regulation of dab1 mRNA and protein in hypothyroid rats**

Recent studies have shown that mutations in the dab1 gene, which encodes an adaptor protein that appears to function in signal transduction processes, lead to cytoarchitectonic alterations similar to those in reeler mutant mice (Sheldon et al., 1997; Howell et al., 1997b; Rice et al., 1998). This suggests that Dab1 acts in the same signaling pathway of Reelin that controls cell positioning in the developing brain. To determine whether dab1 expression was altered in hypothyroid rats, we performed in situ hybridization analyses. At E18 and P0 dab1 transcripts were widely distributed in the proliferative ventricular zone as well as in postmitotic neurons of the cerebral cortex. Both the cortical plate in the neocortex and the pyramidal and granule cell layers of the hippocampus were intensely labeled. In other brain regions such as the cerebellum, widespread expression was also noticed (data not shown), which is consistent with recent studies (Rice et al., 1998). No changes in the distribution of transcripts or the intensity of the hybridization signal were observed in hypothyroid rats at E18–P0 (Fig. 6A,B). At P5–P15 dab1 expression was also widespread in the neocortex and hippocampus with many labeled neurons evident (Fig. 6C,D). In the cerebellum dab1 was expressed in Purkinje cells, in the IGL, and in the inner part of the EGL (data not shown). Again, no major changes were detected in hypothyroid rats, except for a slightly lower signal at P5. These data indicate that dab1 RNA synthesis or stability is not substantially altered by the lack of thyroid hormone.

We next examined the distribution of Dab1 protein, which accumulates to abnormally high level in the absence of Reelin in reeler mice (Rice et al., 1998). At P0, Dab1 immunoreactivity was detected in the perikarya and dendrites of many postmitotic neurons of the neocortex and hippocampus, as well as in fiber tracts in control rats (Fig. 6E; data not shown). At this age, hypothyroid brains showed a similar distribution of Dab1 protein; however, the intensity of immunostaining was higher compared to that in control rats (Fig. 6E,F). At P5, the distribution of Dab1 in the cerebral cortex remained widespread in both control and hypothyroid rats. However, at P5 the levels of immunostaining were higher in control than in hypothyroid rats (Fig. 6, compare G,H). The same difference in Dab1 expression was found in the cerebellum (Fig. 6L,J). At later developmental stages (P15, P25) no differences were seen between controls and hypothyroid rats (data not shown).

To further confirm these data, we analyzed the amount of Dab1 protein in cortex and cerebella from control and hypothyroid animals by Western blotting. As shown in Figure 7, at P5 Dab1 was more abundant in control rats. These results indicate that the levels of Dab1 are inversely correlated with those of Reelin in hypothyroid rats, as recently shown in the reeler mutant mouse (Rice et al., 1998). This also implies that Dab1 fails to be degraded in the absence of a Reelin-evoked signal. Brain samples from wild-type and reeler mice were included as controls, showing the upregulation of Dab1 expression in mutant animals lacking Reelin.

**reelin expression is regulated by thyroid hormone in vivo and in vitro**

To test whether reelin expression is directly regulated by thyroid hormone, organotypic hippocampal slices from control and hypothyroid P0 rats were incubated in culture medium containing either normal serum or T3/T4-depleted serum supplemented or not with T3 (150 or 500 nm). After 6 d in culture, reelin mRNA expression was analyzed by in situ hybridization. Slices from control rats incubated in the T3/T4-depleted or normal serum showed the typical pattern of reelin mRNA-positive neurons. Thus, intensely labeled CR cells were present in the SLM near the hippocampal fissure, and a few additional scattered neurons were present in the remaining layers (Fig. 8A,D; data not shown). Control slices incubated with T3 did not exhibit a statistically significant increase in the number of reelin-positive neurons (Fig. 9). These data suggest that the effect of hormone-depleted serum in vitro may not be as severe as long-term hypothyroidism in vivo.

Slices from hypothyroid newborn rats cultured with either normal or hormone-depleted sera displayed a marked reduction in the number of reelin-positive cells after 6 d (Figs. 8B,E,9). This result was in contrast to the similar number of reelin-expressing cells found in the hippocampus of control and hypothyroid rats at P5 (Fig. 3), and indicates that additional systemic factors regulating reelin expression may exist that are not present in slice cultures. Furthermore, the labeled neurons exhibited weak hybridization signals. In contrast, hypothyroid hippocampal slices treated with T3 displayed a pattern of expression indistinguishable from that of control cultures, indicating that thyroid hormone restores reelin expression to normal levels (Fig. 8C,F). This result was confirmed by counting positive cells (Fig. 9). Additionally, the effect of T3 on reelin RNA expression in the organotypic cultures was estimated by using a semiquantitative RT-PCR analysis. In line with the above data, T3 treatment restored reelin RNA expression in hypothyroid rats to normal levels, and led to a threefold increase in control animals (Fig. 10).

To examine whether the regulation of reelin RNA expression correlated with protein levels, hippocampal slices were immunostained with the CR50 antibody. As seen in Figure 8 (compare panels G and J with H and K) the robust CR50 immunostaining seen in control slices was dramatically reduced in hippocampal slices from hypothyroid rats. Again, the pattern of CR50 immunostaining returned to normal when these cultures were treated with T3 for 6 d (Fig. 8L).
Figure 5. Patterns of reelin RNA and protein distributions in the cerebellum and olfactory bulb of hypothyroid rats. A-F, Distribution of reelin RNA in the cerebellum of control (A, C, E) and hypothyroid (B, D, F) rats. Note the decreased RNA levels in hypothyroid rats at P0, and the opposite increased levels at P5 and P15 in these animals. G-J, Pattern of CR50 immunostaining in the cerebellum of control (G, I) and hypothyroid (H, J) rats at P5 (G, H) and P15 (I, J). Increased Reelin levels are observed in hypothyroid rats. Arrowheads point to the external granule cell layer. K, L, CR50 immunostaining in the olfactory bulb of control (K) and hypothyroid (L) rats at P5, illustrating the decreased immunolabeling in the mitral cells and glomerular neurons in hypothyroid rats. EGL, External granule cell layer; IGL, internal granule cell layer; ML, molecular layer; WM, white matter; MCL, mitral cell layer; GCL, granule cell layer; GL, glomerular cell layer. Scale bars: A, 200 μm (applies to B–J); K, 40 μm (applies to L).
Figure 6. Distribution of dab1 RNA and protein levels in the cerebral cortex and cerebellum of control (C) and hypothyroid (H) rats. A–D, Pattern of dab1 RNA hybridization at P0 and P5 in control (A, C) and hypothyroid (B, D) rats in the hippocampus (A, B) and neocortex (C, D). dab1 is widely expressed within the cerebral cortex, and no major differences are observed between control and hypothyroid rats. E–H, Distribution of Dab1 immunolabeling in the neocortex of control (E, G) and hypothyroid (F, H) rats at P0 and P5. Increased levels of Dab1 immunoreactivity are observed in hypothyroid rats at P0, whereas the opposite occurs at P5. I, J, Photomicrographs illustrating decreased Dab1 immunostaining in the cerebellum of hypothyroid (J) compared to controls (I) rats at P5. Abbreviations are as in legends to Figures 2 and 5. Scale bars: A, 200 μm (applies to B–H); I, 100 μm (applies to J).
results, the pattern of reelin mRNA distribution did not differ greatly at P15 between control and hypothyroid rats, but the intensity of labeling was however reduced in the latter animals (Fig. 11A). Hypothyroid rats treated with T4 showed an increase in the number of reelin-positive neurons present in layer I/SLM and in layers V/VI (Fig. 11B). In addition, T4 treatment resulted in a stronger hybridization signal in labeled neurons. Together, these results indicate that reelin RNA and protein expression are regulated by thyroid hormone in vivo.

DISCUSSION

Reelin and Dab1 are critical for neuronal migration, which in turn is responsible for lamination and precise cellular localization during CNS development. We show here that thyroid hormone, an agent known to exert broad regulatory actions on brain matur-ation (Legrand, 1984; Dussault and Ruel, 1987; Porterfield and Hendrich, 1993), increases reelin RNA and protein levels. The exact mechanism of T3 action on reelin expression, whether transcriptional or post-transcriptional, remains to be determined. The lack of a strict correlation between the changes in RNA and protein levels might indicate that reelin expression is regulated at multiple levels, perhaps by thyroid hormone. Furthermore, the differences observed in the effect of the hormone in distinct brain regions suggests that T3 may cooperate with locally acting factors, or that hormone action is modulated by region- or cell-specific proteins. Recently, BDNF has been found to negatively regulate reelin gene expression. In the present data appear to favor that CR cell number is not altered in the hypothyroid state.

It is interesting to note that in contrast to reelin, dab1 RNA levels are not affected by hypothyroidism. However, Dab1 protein levels are modulated in thyroid-deficient rats. This effect may be caused by a direct effect of T3 on dab1 mRNA translation or on Dab1 protein stability. Alternatively, changes in Dab1 protein content may be an indirect consequence of the reduction in reelin expression by the lack of hormone. In fact, reeler mutant mice show normal dab1 mRNA levels but increased Dab1 protein content (Rice et al., 1998).

The possibility that thyroid hormone primarily controls Dab1 levels and as a consequence affects secondarily the expression of reelin RNA and protein cannot be ruled out, but it does not appear very likely since the expression of Reelin is unaltered in scrambler and yotani mice carrying mutations in the dab1 gene (Yoneshima et al., 1997; Rice et al., 1998), and dab1 RNA expression is not changed in hypothyroid rats. Given the important role of these proteins in migration, it is conceivable that the profound alteration in Reelin/Dab1 levels may affect this process in the hypothyroid brain.

Thyroid hormone exerts its wide regulatory actions by controlling gene expression. In the last years, a number of genes have been described by us and others to be under thyroid control in the CNS (for review, see Bernal and Guadan˜o-Ferraz, 1998). The classical mechanism of action of T3 is the regulation of gene transcription through the binding to specific nuclear receptors (TRα1, TRβ1, and TRβ2 isoforms) that interact with specific nucleotide sequences (T3REs: thyroid response elements) present in target genes, usually in the form of heterodimers with the RXR 9-cis retinoic acid receptor (Lazar, 1993; Muñoz and Bernal, 1997). T3 can regulate gene transcription through the activation of positive T3REs or repression of negative T3REs, or by interference with other transcription factors (Muñoz and Bernal, 1997). In addition, several studies have indicated post-transcriptional regulatory effects of T3 on mRNA stabilization, processing, and translation, or on post-translational mechanisms (Aniello et al., 1991). Besides the above discussed data on BDNF expression, we and others have previously reported a positive regulation of nerve growth factor (NGF), the trkA gene encoding its high-affinity receptor, and neurotrophin (NT)-3 genes by thy-roid hormone during rat brain development (Walker et al., 1982; Lindholm et al., 1993; Alvarez-Dolado et al., 1994). In contrast, abnormally higher levels of the p75NGFR low-affinity receptor for neurotrophins are expressed in the hypothyroid brain (Figuiredo et al., 1993; Alvarez-Dolado et al., 1994). These data are in line with the functional interplay described between thy-roid hormone and NGF in the developing rodent brain and in vitro in pheochromocytoma PC12 cells (Patel et al., 1988; Clos and Legrand, 1990; Muñoz et al., 1993).

Cell migration is known to be altered by hypothyroidism in the neonatal cerebellum (for review, see Legrand, 1984). The migra-tion of cerebellar granule cells from the external to the internal layer is delayed as a consequence of a reduction in their rate of

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**Figure 7.** Western blot analysis of the expression of Dab1 in the cortex (Cx) and cerebellum (Cb) of control (C) and hypothyroid (H) rats at P5. Total brain extracts from wild-type (wt) and reeler (rl⁻/⁻) mice at the indicated ages were used as controls, showing a high increase in Dab1 content in reeler mutants. Numbers to the right indicate Mr of marker proteins.
movement through the molecular layer and Bergmann glia (Lauder, 1979). In addition, ectopic localization of Purkinje cells is a typical abnormality found in the hypothyroid cerebellum, which remarkably also occurs to much higher extent in reeler mice (Mariani et al., 1977; Legrand, 1984; Miyata et al., 1997 and references therein). In contrast, cerebral neuronal migration has been traditionally considered to be unaltered by hypothyroidism, possibly because of the fact that this process is mostly completed.

Figure 8. reelin RNA (A–F) and protein (G–L) expression in hippocampal organotypic slice cultures. Left panels, (A–J). Slices from euthyroid rats incubated for 6 days in standard serum. Middle panels, (B–K). Slices from hypothyroid rats incubated for 6 days in thyroid-depleted serum. Right panels, (C–L). Slices from hypothyroid rats incubated for 6 days in T3/T4-depleted serum supplemented with 500 nM T3. Note that the reduced expression levels in hypothyroid slices are rescued by T3 treatment. Higher magnification photomicrographs illustrating reelin RNA (D–F) and protein (J–L) in the CR cells of the hippocampus are shown. Abbreviations are as in legends to Figure 2; S, subiculum. Scale bars: A, 300 μm (applies to B, C, and G–I); D, 75 μm (applies to E, F); J, 50 μm (applies to K, L)
before birth, and also by the presumption that the fetal brain is insensitive to thyroid hormone (Schwartz et al., 1997). However, recent data have led to a reconsideration of this notion. An abnormal laminar distribution has been reported in the auditory cortex of hypothyroid rats, including an increased number of neurons in layers V/VI, a concomitant decrease in layers II to IV, and the abnormal presence of neurons in the subcortical white matter (Berbel et al., 1993; Lucio et al., 1997). These cytoarchitectonic abnormalities most probably reflect migration defects in the cortex. Also, it has recently been shown that iodine deficiency causes an impaired maturation of hippocampal radial glial cells, which are involved in neuronal migration (Martínez-Galán et al., 1997). Additionally, hypothyroidism affects the migration of cells from germinative zones toward the olfactory bulb and caudate putamen region (Patel et al., 1976; Lu and Brown, 1977). These observations can be linked to the reduction in Reelin content in the hypothyroid brain during the perinatal period reported here. The abnormal expression of Reelin at around birth argues against the proposed thyroid resistance of the fetal brain and clearly indicates that Reelin is an early target of thyroid action during late fetal development.

This work provides the first demonstration that thyroid hormone regulates the expression of Reelin, a gene implicated in the control of neuronal migration. Recently, other mutant mice such as those lacking the cdk5 or p35 genes have been shown to display also migration deficits that disrupt normal cortical lamination (Ohshima et al., 1996; Chae et al., 1997; Kwon and Tsai, 1998). Although the patterns of alterations throughout the brains of these mice are distinct, suggesting that p35/cdk5 and Reelin probably signal through different pathways, our results suggest that it may be interesting to analyze whether cdk5/p35 are under thyroid control.

Our results show that the migration deficits observed in the hypothyroid brain may in part be caused by alterations in Reelin expression. Previously, we reported changes in the expression of other genes such as tenascin-C and neural cell adhesion molecule, which could also contribute to alterations in cell migration (Iglesias et al., 1996; Alvarez-Dolado et al., 1998). The complexity of the processes underlying cell migration (dynamic changes in cytoskeleton and in cell to cell and cell to matrix adhesion, active movement, degradation of extracellular matrix) suggests the existence of multiple sites of possible regulation. Among them, and according to the drastic phenotype caused by its lack of expression, Reelin seems to be clearly relevant. The finding that thyroid hormone influences Reelin expression suggests a molecular mechanism that may be of fundamental importance in understanding the alterations that occur in the hypothyroid brain during development.
Figure 11. Effect of thyroid hormone treatment in vivo on the number of reelin-positive cells present in P15 rats. A, Photomicrographs illustrating the level of reelin RNA expression in the parietal cortex of control (C), hypothyroid (H), and hypothyroid rats treated with T4 (H+T4), as described in Materials and Methods. B, Number of reelin RNA-positive neurons in the neocortex (layers I and V/VI) and hippocampus (SLM) of the three groups of animals. Data were quantitated as described in Materials and Methods (mean ± SEM; *p < 0.05). For cortical layer I and SLM each value corresponds to three strips of four different animals, and for layers V/VI to four sections of three animals. Abbreviations are as in legend to Figure 2. Scale bar, 100 μm.

REFERENCES


