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Maternal Thyroid Hormone before the Onset of Fetal Thyroid Function Regulates Reelin and Downstream Signaling Cascade Affecting Neocortical Neuronal Migration

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Though aberrant neuronal migration in response to maternal thyroid hormone (TH) deficiency before the onset of fetal thyroid function (embryonic day [E] 17.5) in rat cerebral cortex has been described, molecular events mediating morphogenic actions have remained elusive. To investigate the effect of maternal TH deficiency on neocortical development, rat dams were maintained on methimazole from gestational day 6 until sacrifice. Decreased number and length of radial glia, loss of neuronal bipolarity, and impaired neuronal migration were correctible with early (E13–15) TH replacement. Reelin downregulation under hypothyroidism is neither due to enhanced apoptosis in Cajal–Retzius cells nor mediated through brain-derived neurotrophic factor–tyrosine receptor kinase B alterations. Results based on gel shift and chromatin immunoprecipitation assays show the transcriptional control of reelin by TH through the presence of intronic TH response element. Furthermore, hypothyroidism significantly increased TH receptor α1 with decreased reelin, apolipoprotein E receptor 2, very low–density lipoprotein receptor expression, and activation of cytosolic adapter protein disabled 1 that compromised the reelin signaling. Integrins (α_v and β_1) are significantly decreased without alteration of α_3 indicating intact neuroglial recognition but disrupted adhesion and glial endfeet attachment. Results provide mechanistic basis of essentiality of adequate maternal TH levels to ensue proper fetal neocortical cytoarchitecture and importance of early thyroxine replacement.

Keywords: development, integrin, neocortex, neuronal migration, reelin

Introduction

Radial migration of neurons to cortical plate in the developing rat cerebral cortex reaches near completion by embryonic day (E) 18. Adequate levels of maternal thyroid hormone (TH) before the start (E17.5) of fetal thyroid function (FTF) are considered to be essential for optimal neuronal migration and spatiotemporally formed morphogenic pattern of neocortex. Aberrant cell migration, ectopic location of neurons, and abnormal brain lamination are of special relevance to associated mental retardation and neurological deficits (Aicardi 1994; Auso et al. 2004). In the developing cerebral cortex, migration of neurons depends on supporting radial glial scaffold as well as reelin secretion from Cajal–Retzius (CR) cells to form the respective layers of the neocortex (Rakic 1990). How the number, elongation, and orientation of radial glia as well as molecular cues emanating from both the cell types are affected by maternal hypothyroidism alone during development is not known. The mechanisms involved probably encompass interplay between TH, extracellular cues, and intracellular signaling. In humans as well as in rodents, THs and their receptor (TR) are present in the fetal cerebral cortex prior to the onset of FTF (Bernal and Pekonen 1984; Obregon et al. 1984; Contempré et al. 1993; de Escobar et al. 2004, 2008).

Reelin serves 2 important functions for neuronal migration, namely, 1) the state of readiness of neurons to migrate and 2) upward movement of neurons on radial glial scaffold (Rice and Curran 2001; Uchida et al. 2009). These functions are achieved by binding with apolipoprotein E receptor 2 (ApoER2) and very low–density lipoprotein receptor (VLDLR) on cell surface and subsequent phosphorylation of cytosolic adapter protein disabled 1 (Dab1) (Howell et al. 1997, 1999; D'Arcangelo et al. 1999; Dulabon et al. 2000). The formation of various integrin receptor heterodimers like $\alpha_3\beta_1$ and $\alpha_v\beta_1$ modulates the recognition, attachment, and detachment of neurons from the glial tracts (Anton et al. 1999; Dulabon et al. 2000).

The question whether reduced reelin expression seen under hypothyroid condition is caused by the loss of CR cells or through alterations in brain-derived neurotrophic factor (BDNF)–tyrosine receptor kinase B (trkB) pathway has not been addressed. The lack of agreement on subpopulations of emerging reelin-expressing CR neuron responsive to TH during neocortical development is difficult to fathom and needs to be addressed (Alvarez-Dolado et al. 1999). Furthermore, since TH controls gene expression by interacting with its nuclear receptors (Oppenheimer and Schwartz 1997; Forrest and Vennström 2000; Yen et al. 2006), the assumption of a putative thyroid hormone response element (TRE) in the *reelin* gene is yet to be experimentally confirmed.

Though reelin and Dab1 have been identified as targets of TH action during perinatal neocortical development (Alvarez-Dolado et al. 1999), the effect of maternal TH deficiency on reelin signaling pathways and their significance on fetal cerebral cortex development has not been elucidated.

The results document reduction in neuroglial numbers and change in their morphology as a consequence of maternal hypothyroidism effecting retarded neuronal migration reversible only through early replacement of thyroxine. Furthermore, reduced reelin levels are neither due to apoptotic loss of CR cells nor mediated through BDNF–trkB alterations at E16. The first time demonstration of in vivo binding of TR to a specific intronic TRE present in reelin gene proves its direct regulation by TH. Diminished ontogenic expression of reelin, its receptors ApoER2, VLDLR, integrins, and phosphorylation of downstream Dab1 indicate a multifactorial effect of maternal TH deficiency that precedes FTF.

Materials and Methods

Animals and Treatments

All animal procedures performed above were approved by the Institutional Guidelines for Animal Care and Research. Sprague–Dawley

rats were housed in 12-h day and night cycle environment with ad libitum availability of chow diet and tap water. The day of visualization of spermatozoa in vaginal smears was designated as E0. The pregnant rats were divided into euthyroid (n = 15) and hypothyroid (n = 28) groups. To induce hypothyroidism, 2-mercapto-1-methylimidazole (0.025% w/v; Sigma) was given to pregnant rats in drinking water from gestational day 6 and continued until the animals were sacrificed. Total thyroxine (TT4) and total triiodothyronine (TT3) were measured in the serum of the pregnant dams prior to dissection by radio immuno assay using DPC kits. This protocol ensures that animals are hypothyroid as shown by us earlier (Sinha et al. 2008). For reversibility experiments, hypothyroid dams (n = 3 each) were injected with a single daily dose (1.5 μg/100 g body weight) of T4 intraperitoneally (i.p.) from E13 to E15 (early treatment) and from E17 to E19 (late treatment). This restored the serum TH levels of pregnant dams and postnatal pups equivalent to age-matched euthyroid controls.

Animals were sacrificed at different embryonic and postnatal (P) stages, and whole brains were fixed in 4% paraformaldehyde. Paraffin-embedded sections containing the somatosensory region of the cerebral cortex were cut in the coronal plane. Dewaxed sections were rehydrated and used for different staining protocols.

Hematoxylin and Eosin Staining

Sections from P24 stage pups (n = 5 each, for euthyroid, hypothyroid, and early and late reversibility groups from different litters) were stained with Harris's hematoxylin and washed in running tap water followed by acid alcohol wash for 5–10 s. Sections were counterstained in eosin and washed in tap water for 15–30 s. The sections were then dehydrated through gradient alcohol series, cleared in xylene, and mounted in Di-n-butylPhthalate in Xylene mountant.

BrdU Labeling and Analysis

Pregnant rats (n = 5 each, for euthyroid, hypothyroid, and early reversibility group) were injected i.p. with 50 mg/kg body weight bromodeoxyuridine (BrdU; Sigma) at E16 and E17 in a single daily dose. The pups born to these dams were dissected at P24, and paraffin-embedded sections were prepared. After antigen retrieval, sections were blocked in 5% normal sheep serum containing 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 60 min at room temperature, followed by incubation in mouse anti-BrdU antibody (1:200; Santa Cruz Biotechnology, Inc.) overnight at 4 °C. Sections were stained with 3,3-diaminobenzidine peroxidase (DAB)/H_2O_2 as chromogen (Quick Universal ABC KIT, Vector Laboratories). The distribution of BrdU-positive cells was counted in the different compartments of layers II/III, IV, and V/VI, and percentage of BrdU-labeled cells in each area was determined, and results were plotted as histogram.

Immunostaining

Antigen retrieval of sections was done in boiling citrate buffer (10 mM, pH 6.0). After quenching the autofluorescence with 1% sodium borohydride in dark for 30 min at room temperature, sections were incubated in blocking buffer and then with respective antibodies: doublecortin (DCX; 1:2000; Abcam), vimentin (1:200; Santa Cruz Biotechnology, Inc.), reelin (1:1000; G10; kind gift from Dr Andre M. Goffinet), calretinin (1:500; BD Transduction Laboratories), and Dab1 pY220 (1:100; Abcam) in 5% normal sheep serum overnight at 4 °C. Next day, after washing with PBS, the sections were incubated with fluorochrome-tagged respective secondary antibodies for 1 h in dark. The sections were counterstained with Hoechst 33258 (Molecular Probes) nuclear stain and mounted in antifade mountant. The stained sections were observed, and images were collected with a Zeiss Laser Confocal Scanning Microscope 510 META (Zeiss).

For double immunostaining by reelin (G10) and TH receptor α1 (TRα1; Abcam), sections were stained with DAB/H_2O_2 and Vector Red (Vector Laboratories) as per manufacturer's protocol.

TUNEL Assay

In situ detection of apoptosis was performed at E16 stage by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) kit (Roche Molecular Biochemicals) according to manufacturer's instructions. TUNEL-positive cells were counted in 5 randomly selected fields, spanning the marginal zone (MZ). Relative TUNEL positivity was expressed as number of TUNEL-positive cells/100 nuclei (Hoechst stained). Image-Pro Plus 5.1 software was used for image capturing and cell counting (Media Cybernetics, Inc.).

RNA Extraction and Analysis

Total RNA was isolated from the fetal neocortex at E14, E16, and E18 by single-step method using TRIZOL reagent (MRC). Two micrograms of total RNA was reverse transcribed using cDNA Archive kit (Applied Biosystems). Real-time analysis of BDNF, trkB, and normalizing gene, gapdh, was performed using specific Taqman UNIVERSAL PCR MASTER mix assays (Rn00560868_m1 [BDNF], Rn00820626_m1 [trkB], Rn00576699_m1 [gapdh]) as per the manufacturer's instructions (Applied Biosystems), and fold changes in gene expression were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Semiquantitative reverse transcription (RT)–polymerase chain reaction (PCR) was used to analyze reelin expression using gapdh as an internal control. The primers are listed in Supplementary Table 2.

Gel Shift Assay

Nuclear extract was prepared from E16 cerebral cortices from the euthyroid rat fetuses prior to gel shift assay. The direct repeat 4 (DR4) site containing oligonucleotides (5′-ACGAGGTCACAGGAGGTCACACAAG-3′) was annealed in the buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) and labeled by T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-32P]adenosine triphosphate. The labeled probes were purified using Sepharose G25 column. Binding reactions were performed for 20 min using the radiolabeled DNA probes (20 000 c.p.m.) and the nuclear lysate (1 μL) in 10% glycerol, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 30 mM KCl, 4 mM spermidine, 0.1 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, and poly(dI-dC) (1.5 μg). Unlabeled competitor oligonucleotides were included at the indicated molar excess in the binding reactions. Where indicated, anti-TRα1 antibody (Santa Cruz Biotechnology, Inc.) has been included in the reaction mix for 30 min on ice. After binding, reaction samples were electrophoresed on a 6% non-denaturing polyacrylamide gel, followed by gel drying and exposure to X-ray film.

Chromatin Immunoprecipitation and DNA Analysis

The chromatin immunoprecipitation (ChIP) assay was performed on single cells prepared from the freshly dissected E16 neocortices. In brief, cross-linking was done by incubation with 1.5% formaldehyde for 15 min at room temperature. The reaction was stopped by the addition of glycine to a final

concentration of 125 mM, followed by 2 washes of cold PBS (Invitrogen) containing protease inhibitors (Sigma). Thirty-five micrograms of sonicated DNA (fragments of 200–1000 bp, by using a Misonix XL-2000 sonicator) was incubated with 50 μL of anti-TRα1 antibody (Santa Cruz Biotechnology, Inc.) and anti-cardiac trponin T (CTnT; Serotech) antibody overnight at 4 °C. At the end of the reaction and after washing steps, DNA was extracted by phenol–chloroform and suspended in 30 μL of H_2O. The precipitated DNA from these different antibodies and in the starting inputs was then analyzed by conventional PCR to reveal the presence of specific DNA templates. Four microliters of samples were used for conventional PCR according to manufacturer's instructions (Qiagen) using primers listed in Supplementary Table 2.

Western Blotting

Cerebral cortices from different stages (E14, E16, E18, and E20) from both the euthyroid and the hypothyroid groups were homogenized in lysis buffer (10 mM Tris–Cl, pH 7.5, 50 mM NaCl, 1% Triton X-100 containing phenylmethylsulfonyl fluoride [1 mM] and protease inhibitor cocktail), centrifuged at 12 000 × *g* for 15 min at 4 °C, and collected supernatant was estimated for protein content. Hundred micrograms of proteins were subjected to 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membrane. The membranes were incubated with anti-ApoER2, anti-VLDLR (kindly gifted by Dr J. Nimph), anti-Dab1 (kindly gifted by Dr J. Herz), anti-α_3 integrin (kindly gifted by Dr M. Dipersio), anti-β_1 integrin (Abcam), anti-α_v integrin (kindly gifted by Dr L. Reicherdt), or anti-β-actin (Santa Cruz Biotechnology, Inc.) antibodies followed by incubation with horseradish peroxidase (HRP)–conjugated corresponding secondary antibodies. For Dab1 pY220 (Abcam) immunoblotting, 200 μg protein was first immunoprecipitated with Dab1 antibody, followed by immunoblotting with anti-Dab1 pY220 antibody and subsequently incubation with HRP-anti-rabbit secondary antibody. The signals were detected using an enhanced chemiluminescence detection system (Amersham Biosciences). Relative expression of each protein was determined by densitometric analysis using LabWorks 4.0 software (UVP Ltd).

Statistical Analysis

All the experiments were repeated with 5 different litters. Statistical analysis was performed by using SPSS software version 11. The data are presented as a mean ± standard error. Significant differences between groups were compared by 2-way analysis of variance (ANOVA), factors being developmental stages and experimental groups (euthyroid vs. hypothyroid). One-way ANOVA was then used to identify developmental stages affected by the hypothyroidism, followed by Tukey's or Duncan post hoc test. P value <0.05 was considered statistically significant.

Results

Induction and Assessment of Hypothyroidism in Pregnant Dams and Their Offsprings

Serum THs (both TT4 and TT3) showing significant increase with gestational age in euthyroid dams registered a progressive significant decrease ($P < 0.01$) in TH deficiency group indicating achievement of hypothyroidism in methimazole-treated dams (Supplementary Table 1). This corroborated well with associated clinical features characteristic of hypothyroidism such as retarded weight gain, delayed eye opening, and poor pelage development in the pups (Supplementary Fig. S1). In the reversibility group, hypothyroid dams ($n = 5$ each) injected with a single daily dose (1.5 μg/100 g body weight) of T4 i.p. from E13 to E15 (early treatment) and from E17 to E19 (late treatment) showed restoration of the serum TH levels of pregnant dams and postnatal pups equivalent to age-matched euthyroid controls (Supplementary Table 1).

Maternal Hypothyroidism Affects Neocortical Cytoarchitecture and Neuronal Migration

The hematoxylin and eosin–stained coronal sections of P24 stage pups showed marked difference in cytoarchitecture of somatosensory region of cerebral cortex under TH deficiency during the critical window period of corticogenesis and neuronal migration that takes place before FTF. Typical barrels in layer IV indicated by the arrows and distinguishable layer borders seen in euthyroid pups were absent under hypothyroid condition (Supplementary Fig. S2*A,B*). This is correctible only on early treatment (E13–E15) but not on late treatment (E17–E19) with T4 (Supplementary Fig. S2*C,D*). Cell density was higher in deeper layers (layers V–VI) and white matter under TH deficiency. The pyramidal neurons in cortex of hypothyroid pups were smaller with stunted process formation as shown in inset (Supplementary Fig. S2).

The aberrant migration of neurons under maternal TH deficiency was also confirmed by radial distribution of BrdU-immunoreactive cells at P24. In euthyroid pups, ~70% immunoreactivity was present in layers II–III (Fig. 1*A,D*). Under hypothyroidism, their proportion decreased significantly ($P < 0.05$) in layers II–III and increased in layers V–VI and white matter (Fig. 1*B,D*). The number of BrdU-immunoreactive cells in euthyroid and in T4 reversibility group cortex (Fig. 1*C,D*) was comparable as reported earlier (Auso et al. 2004).

Maternal Hypothyroidism Disrupts Neuroglial Density and Morphology

Vimentin immunofluorescence at E16 showed reduction in radial glia density as well as stunting under hypothyroid condition (Fig. 2*B*, euthyroid in Fig. 2*A*). Similarly, decrease in DCX-expressing neurons showed loss of migration competence and subsequent thinning of cortical plate under TH deficiency (Fig. 2*E,H* and euthyroid in Fig. 2*D,G*). Higher magnification picture depicted change from bipolar to multipolar neuronal morphology as a consequence of hypothyroidism (Fig. 2*K*, euthyroid in Fig. 2*J*). Inhibition of DCX expression has been linked to the loss of neuronal bipolarity and radial migration in rat neocortex (Bai et al. 2003). The glial cell density, their morphology, as well as neuronal bipolarity and DCX immunoreactivity were regained at E16 on T4 supplementation to hypothyroid dams at E13–E15 (Fig. 2*C,F,I,L*).

Maternal Hypothyroidism Decreases Reelin Levels and CR Cells

It has been known that reelin influences radial glia and regulates neuronal migration (Förster et al. 2002; Hartfuss et al. 2003). To address whether reelin is the critical molecular cue being affected under maternal hypothyroidism before FTF,

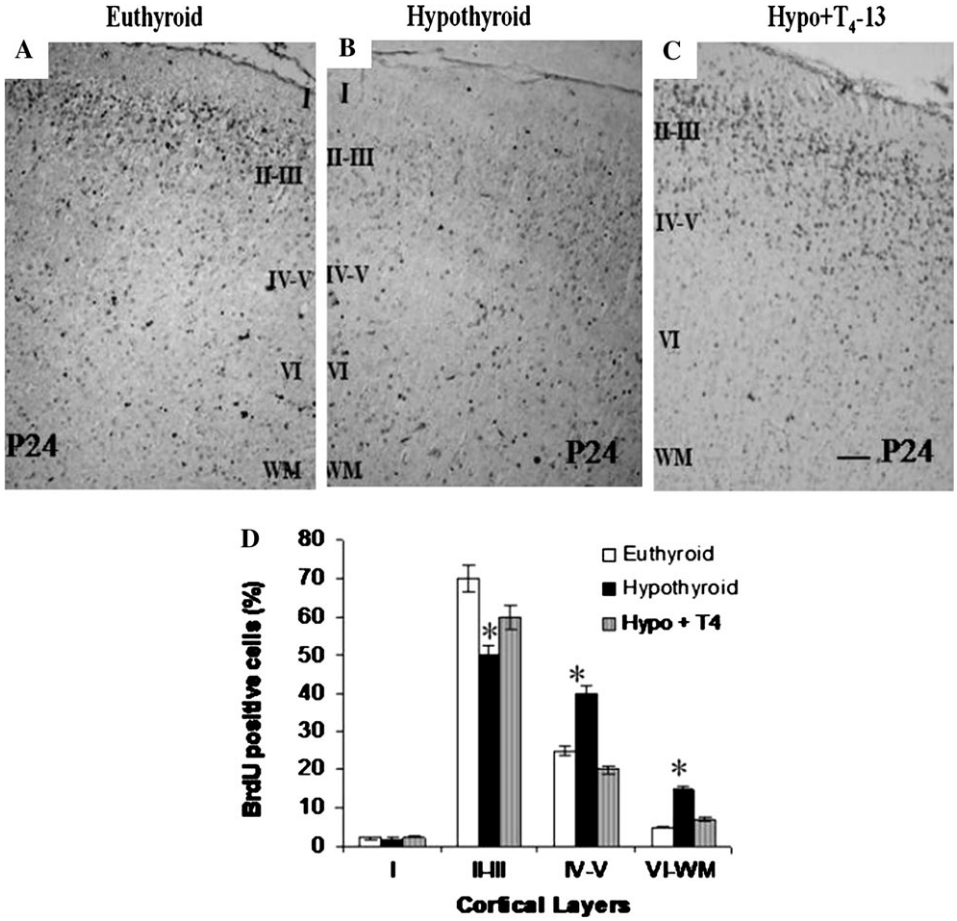

Figure 1. Defects in neuronal migration under maternal hypothyroidism. (*A–D*) BrdU-labeled neurons analyzed at P24 stage accumulated in layers V–VI and white matter under maternal TH deficiency. There were fewer labeled cells in the layers II–III of the hypothyroid pups neocortex (*B*) compared with euthyroid group (*A*), upon early T4 treatment at E13 in Hypo + T4-13 group (*C*), the number of BrdU-labeled cells are comparable with euthyroid group. For Hypo + T4 group, T4 was injected (i.p., single daily dose) at E13–E15 to hypothyroid pregnant dams and pups born to them were dissected at P24. (*D*) The histogram shows the distribution of BrdU-positive cells when labeled at E16 and E17 and analyzed at P24 in the different layers of primary somatosensory cortex as a way to quantify radial migration. Asterisks indicate significant differences in percentages of BrdU-positive cells in a given layer in hypothyroid and euthyroid cortices ($n = 5$; $^*P < 0.05$) (scale bar: 100 μm).

we investigated spatiotemporal distribution of reelin. Reelin-positive CR neurons lined the MZ (layer I) of the neocortex, and age-matched comparison revealed a significant decrease in their number under hypothyroidism at all stages from E14 ($P < 0.05$) till E18 ($P < 0.01$) (Fig. 3*A–G*) that reverses on T4 treatment (Fig. 3*J–L*). CR neurons also display weak reelin intensity under TH deficiency (Fig. 3*H,I*). The diffuse staining below the MZ seen is likely to correspond to the distribution of extracellular reelin secreted from CR cells. Significant decrease in CR neurons at E16 under hypothyroid condition was further confirmed through calretinin immunofluorescence (Supplementary Fig. S3*A–D*).

To identify the reason for decrease in reelin-positive CR cells under hypothyroidism, TUNEL assay was done at E16 stage. Unaltered number of TUNEL-positive cells in MZ excludes the possibility of enhanced apoptosis under maternal hypothyroidism (Supplementary Fig. S3*E–G*).

Effect of TH Deficiency on BDNF and trkB Expression

Since BDNF, a TH responsive gene that acts through trkB receptor, is a negative regulator of reelin expression in CR cells (Ringstedt et al. 1998), we next investigated BDNF and trkB messenger RNA levels. Though the real-time PCR analysis showed significantly reduced ($P < 0.05$) BDNF and increased trkB expression in hypothyroid fetuses at E14 stage, insignificant alteration at E16–E18 period (Fig. 4*A,B*) suggests reelin downregulation to be independent of BDNF action at least during the critical period of neuronal migration from E16 to E18.

Direct Regulation of Reelin by TH and Evidence of a TRE in reelin Gene

As a first step toward elucidation of direct effect of TH deficiency at E16, we confirmed significantly reduced levels of reelin transcript by RT–PCR (Fig. 4*C,D*). These changes are in conformity with reduced reelin-positive CR neurons seen under hypothyroidism at E16 (Fig. 3*B,E*). THs are known to regulate gene expression through nuclear receptors. Because of reported high expression of TRα1 in cortical plate of developing rat neocortex at E15.5 (Bradley et al. 1992), we looked into its status under maternal TH deficiency. Increased TRα1 expression seen by us under TH deficiency (Fig. 4*E,F*) is in consonance with earlier report (Sinha et al. 2008). This along with colocalized reduced reelin expression (Fig. 4*G,H*) suggested that reelin gene may be a direct target of the TRα1.

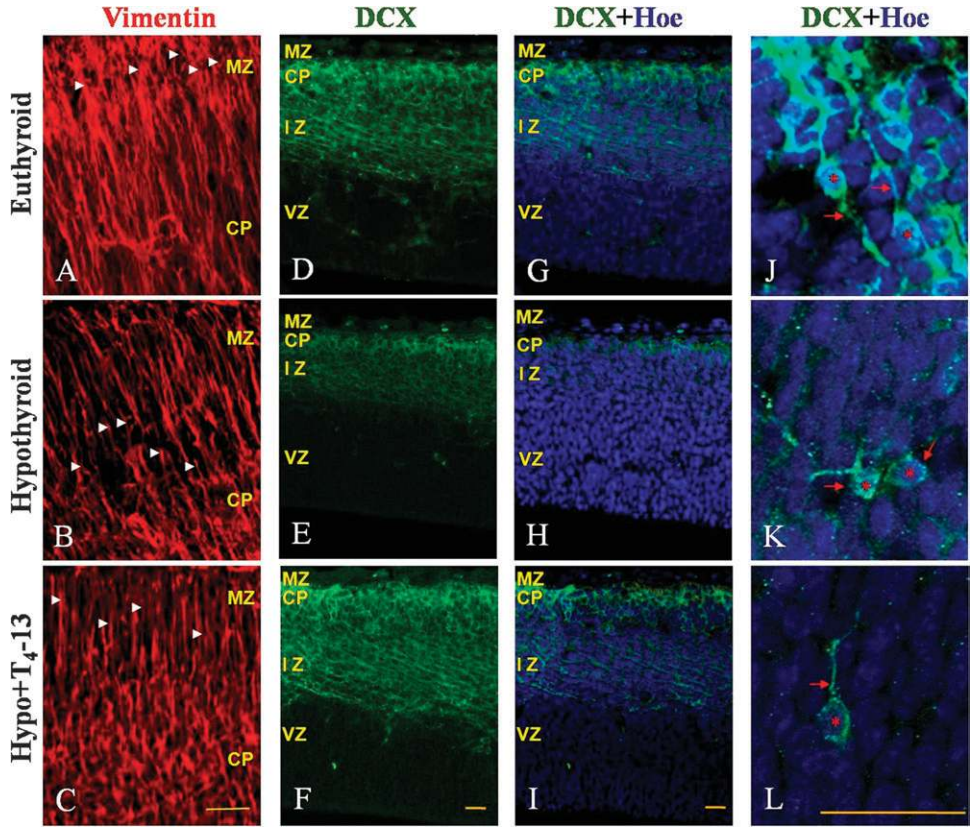

Figure 2. Confocal microscopy shows altered morphology and decreased cell number of radial glia and migrating neuron at E16 ($n = 5$). (*A–C*) Vimentin immunofluorescence of the radial glial cell fibers in the cortical plate (CP) of euthyroid, hypothyroid, and T4 reversible group. In hypothyroid state, the number and length of radial glial fibers are reduced (*B*) (arrowheads) as compared with vertical straight projection pattern of fibers extending throughout the full thickness of CP and reaching above the MZ in euthyroid CP (*A*). The number and morphology of the radial glial cells restored to normal in the TH replacement group (*C*). For Hypo + T4 group, T4 was injected (i.p., single daily dose) at E13–E15 and fetuses were dissected at E16. (*D–L*) DCX immunofluorescence in the cerebral cortex of euthyroid, hypothyroid, and T4 replacement group at E16. Diminished DCX expression seen in intermediate (IZ) and CP of the fetuses from hypothyroid dams (*E*, *H*) as compared with euthyroid fetuses (*D*, *G*). Expression restored to euthyroid levels in the T4 replacement group (*F*, *I*). (*D–L*) Hoechst (Hoe) is used as a nuclear counter stain. (*J–L*) Morphology of DCX-positive neuron as observed under higher magnification (100×), red arrowheads indicate a single cell with cellular processes and the cell soma is shown by asterisks. Neurons depict bipolar shape in the IZ of euthyroid neocortex (*G*) and multipolar morphology in hypothyroid state (*H*) that reverts to bipolar morphology upon T4 replacement (*I*) (scale bars: 20 μm).

In silico prediction of TRE sequences in the rat *reelin* gene using NUBIScan and NHRscan computational search (Podvinec et al. 2002; Sandelin and Wasserman 2005) indicated the presence of a DR4 site in TRE at nucleotide base pairs numbered "+47816-47831" located in intron 1 of *reelin* gene. Gel shift assay results confirmed the presence of a DR4-binding site suggesting the direct regulation of *reelin* gene by TH (Fig. 5*B*). The specificity of the binding was confirmed by annulment of the band shift on addition of TRα-specific antibody. To check whether this TRE present in intron 1 (TRE-int1) is a binding site for TRα in vivo, we performed ChIP assay. Different primer pairs were synthesized; reln1 encompassing the putative TRE region and reln2 specific for region distant from TRE as shown in the schematic diagram (Fig. 5*A*). The sonicated chromatin was incubated with anti-TRα or anti-CTnT antibody. The precipitated DNA and the starting inputs were then analyzed by conventional PCR to reveal the presence of specific DNA templates. While the sonicated DNA containing reelin template sequence gets amplified by both reln1 and reln2 primers (Fig. 5*C*, lane 3, both panels), it is only the TRα-binding site–specific sequence that gets amplified by use of reln1 but not by reln2 primers (Fig. 5*C*, lane 4, both panels). These data clearly demonstrated that, in embryonic neocortical cells in vivo, TRα binds to the *reelin* gene with true TRE-int1.

Maternal Hypothyroidism Compromises Reelin Signaling

To understand how the hypothyroidism-mediated reelin downregulation contributes toward aberrant neuronal migration, we examined the expression of ApoER2, VLDLR, and Dab1. Under TH deficiency, ApoER2 showed significantly reduced levels ($P < 0.05$) till E18 and significantly higher peak value is reached only at E20 (Fig. 6*A*,*D*). VLDLR expression on the other hand remains significantly decreased in hypothyroid group ($P < 0.05$) compared with age-matched euthyroid controls (Fig. 6*B*,*E*). In contrast, low Dab1 presence seen till E16 that increases till E20 in euthyroid cortex showed significantly increased accumulation ($P < 0.05$) under hypothyroid condition (Fig. 6*C*,*F*). Subsequent examination of Dab1 activation status showed reduced level of phosphorylated Dab1 immunofluorescence when stained with Dab1 pY220 antibody (Fig. 6*H*). This finding corroborated by western blot (Fig. 6*I*) suggested its reduced turnover under hypothyroid condition.

Maternal TH Insufficiency Downregulates α_v and β_1 but Not α_3 Integrin Expression

In the context of reelin downregulation, we also examined the expression of various integrins suggested to be essential for the process of neuroglial recognition (α_3), attachment, and its

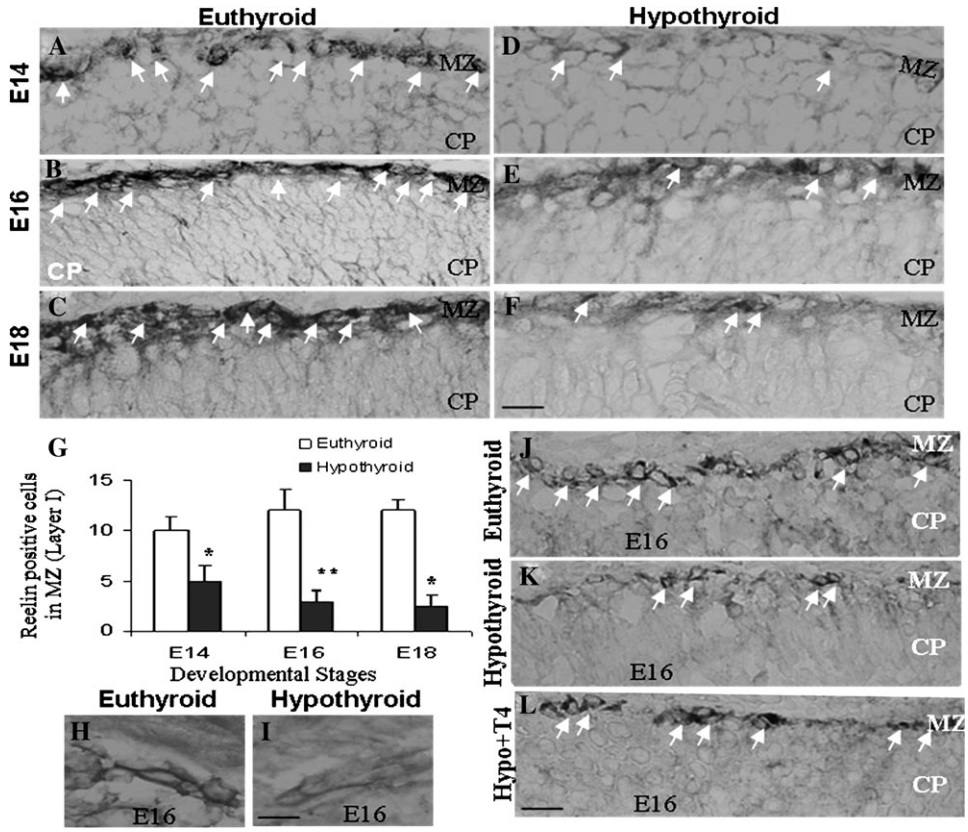

Figure 3. Effect of maternal hypothyroidism on reelin expression in CR cells in the MZ. (*A–G*) Ontogenic expression of reelin in MZ of euthyroid (*A–C*) and hypothyroid (*D–F*) neocortex from E14 to E18. Number and intensity of reelin-producing cells were decreased at all stages in the hypothyroid group (*D–F*, *I*) as compared with euthyroid (*A–C*, *H*). The histogram depicts the density of reelin-positive neurons in MZ (*G*); in the hypothyroid fetal rat neocortex, there is a significant decrease in CR cell density as compared with euthyroid group ($P < 0.05$) at E14 and ($P < 0.01$) at E16–E18. Higher magnification picture shows typical horizontal morphology of the CR cells with reduced reelin intensity in the cells under hypothyroidism (*I*). (*J–L*) Upon T4 supplementation (*L*), the number of reelin-positive cells is comparable with euthyroid (*J*) (scale bars *A–F* and *J–L*: 50 μm; *H–I*: 20 μm).

maintenance (α_v) along with glial end-feet attachment (β_1) located above MZ (Anton et al. 1999; Graus-Porta et al. 2001). Unaltered α_3 integrin expression levels throughout the developmental period irrespective of TH status (Fig. 7*A,D*) exclude radial glial recognition as a hindrance for neuronal migration under hypothyroid condition. However, significantly reduced levels of α_v and β_1 integrin expression ($P < 0.05$) in hypothyroid group during migration phase of E14–E16 seen by us (Fig. 7*B,C,E,F*) point toward possible defective neuroglial attachment necessary for neurons to migrate on radial glial scaffold.

These data clearly demonstrate that maternal hypothyroidism–mediated cytoarchitecture defects including mispositioned neurons, altered morphology, and density of radial glia and loss of bipolarity of migrating neurons can be attributed at least in part to reelin signaling cascade deficits. Moreover, direct control of reelin expression by TH through the presence of TRE has also been proved for the first time.

Discussion

Our analysis of the rat fetal cerebral cortex from hypothyroid dams has revealed suboptimal neocortical cytoarchitecture resulting from TH deficiency prior to E16. This period precedes onset of FTF, and fetal brain is totally dependent on maternal TH supply. Hypothyroidism diminishes the numbers of radial glial scaffold, bipolar neurons, and CR cells that are crucial for inside-out neuronal migration in the cerebral cortex (Figs 2 and 3). In addition, radial glia showed stunting and neurons displayed loss of bipolarity resulting in aberrant neuronal migration. Bai et al. (2003) have reported that bipolar morphology is crucial for neurons to migrate in radial direction to form the cortical plate. The results pertaining to radial glia are in line with the findings of others seen at perinatal period (Martinez-Galan et al. 2004). Our results truly represent the neurological insult exclusively due to maternal hypothyroidism since the thyroxine treatment before the onset of FTF effectively restores cytoarchitecture to the extent seen under euthyroid condition and the treatment after the start of FTF fails to do so. Though the effect of maternal TH on gene expression in fetal rat brain was identified, the molecules involved in neuronal migration were not studied (Dowling et al. 2000).

Maternal TH Availability Affects Molecular Cues for Neuronal Migration

The significant downregulation of reelin seen under TH deficiency was restored on T4 treatment suggesting a relationship between thyroidal status and molecular cues that governs neuronal migration (Fig. 3). In the developing cerebral cortex, reelin-secreting CR cells are required to maintain the radial glial phenotype and to anchor radial glial processes to the MZ (Supér et al. 2000; Hartfuss et al. 2003). Absence of reelin causes, in addition to mispositioning of neurons, the defasciculation of

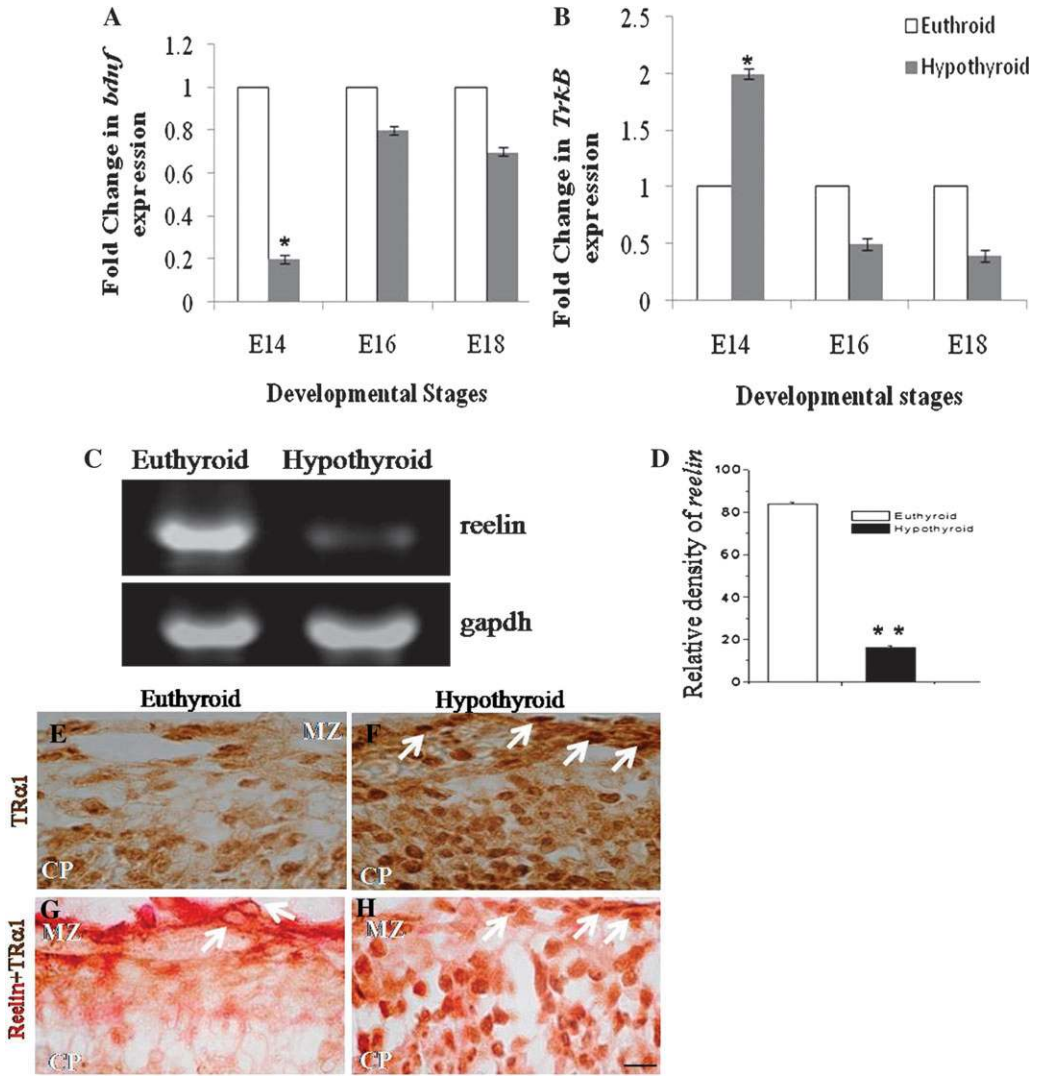

Figure 4. Effect of TH deficiency on BDNF and trkB expression and reelin transcription. (*A*, *B*) Real-time PCR analysis of BDNF (*A*) and trkB (*B*) messenger RNA (mRNA) levels at E14–E18 in developing neocortex. Changes were found to be significant under hypothyroid state only at E14. (*C*, *D*) RT–PCR for reelin mRNA expression; gapdh mRNA was used as an internal control. Densitometric analysis of reelin mRNA expression showed highly significant ($P < 0.01$) decrease in reelin mRNA levels (*D*). (*E*, *F*) TRα1 immunolocalization in cortical plate (CP) and MZ at E16 showing its increased levels under hypothyroid condition in CP and MZ (arrows) (*F*). (*G*, *H*) TRα1 (DAB, brown) and reelin (Vector Red, pink) colocalization in the MZ (arrows) showing an association of increased TRα1 expression with suppressed reelin levels under hypothyroidism (*H*). The antibody used is specific for TRα1 as no signal was obtained in immunohistochemistry after incubation of antibody with the respective peptide as has been provided by Abcam for this antibody (ab 53729) (scale bar *E*–*H*: 20 μm).

radial glial fibers and malformation of their apical processes (Förster et al. 2002). Localization of the components of the reelin signaling cascade in radial glial confirmed that reelin signaling is also present in radial glia (Hartfuss et al. 2003; Luque et al. 2003). The precisely regulated expression of reelin receptors strongly suggests that the primary reelin action occurs on projection neurons at their early/pre-migratory stage in developing cerebral cortex (Uchida et al. 2009).

Similar to our results showing reelin downregulation, others have also shown that TH deficiency affects reelin expression, though at later stages of development (Alvarez-Dolado et al. 1999). In affirmation with suggestion of others, we reconfirmed the reduction of CR cell numbers in TH-deficient neocortex by using calretenin as another marker of CR cells (Alvarez-Dolado et al. 1999; García-Moreno et al. 2007). However, recent electrophysiological study results display many CR subpopulations that may vary in coexpression of reelin, calretenin, as well as sodium channel isoforms. It has been argued that at early stages of development, reelin-positive neurons are not true CR cells but rather a precursor population, termed subpial granule neurons, that is the precursor to postnatal CR cells (Albrieux et al. 2004). In vitro experiments confirming increase in reelin-expressing cells in response to T3 replacement argues in favor of possible presence of non–reelin-expressing cells under TH deficiency (Alvarez-Dolado et al. 1999). Therefore, reduction in CR cell numbers as assessed in the present study may not fully account for all the subpopulations of CR. It necessitated to further check the effect of TH deficiency in terms of reelin gene at transcription and posttranscription level. Our results confirmed the reduction in reelin expression and apparent decrease in CR cell numbers.

We assessed various possible causes of reelin downregulation. The unaltered numbers of TUNEL-positive cells in MZ rules out the possibility of enhanced apoptosis of CR cells in

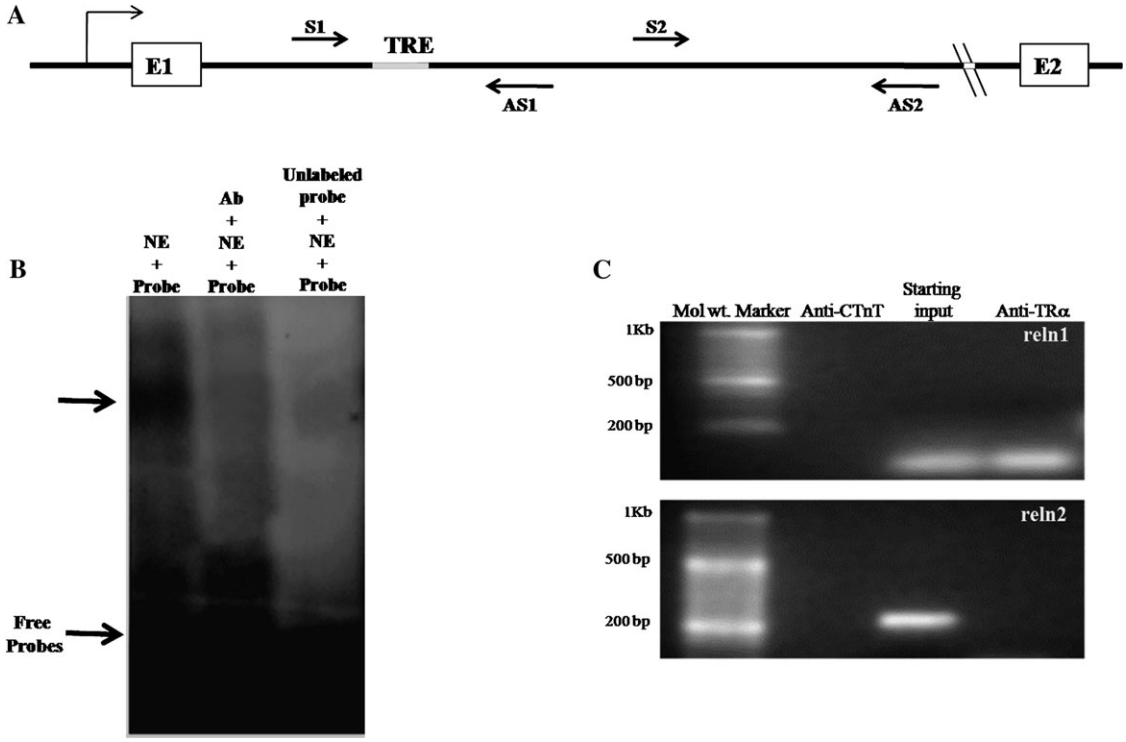

Figure 5. Molecular regulation of reelin by TH. (*A*) Scheme of intron 1 of the *reelin* gene displaying different primers used in the study. Sense (S) and antisense (AS) numbers refer to oligonucleotide primers of the respective reelin-amplified fragment numbers. E1 and E2 refer to exons 1 and 2, respectively. Note that reln1 primers amplify DNA fragments comprising the TRE. reln2 was used as a negative control as the amplified fragments are away from the TRE. (*B*) Gel shift assay showing TR binding to *reelin* gene resident DR4 sequence in nuclear extract of E16 neocortex. Among neocortical nuclear extract, the shift of band is most prominent in lane 1 (upper arrow), whereas no such shift was evident under incubation with either anti-TRα antibody (lane 2) or 100-fold excess unlabeled oligos (lane 3). (*C*) In vivo ChIP study by conventional PCR of the DNA purified from the different samples before and after ChIP. The picture is representative of the results from 3 independent experiments. Within each panel, the fragment amplified on the *reelin* gene is indicated. CTnT antibody was used as a negative control for immunoprecipitation (upper and lower panel, lane 2); the upper panel shows the amplification product (151 kb) from reln1 primer sequence, while the lower panel depicts the amplification product (200 kb) from reln1 primer sequence. Note the presence of amplification product only in reln1 primer sequence in the TRα immunoprecipitated sample (upper panel, lane 4) and its absence in reln2 primers (lower panel, lane 4); the starting input DNA before immunoprecipitation was used as a positive control (upper and lower panel, lane 3).

the embryonic brain under hypothyroid state. How the TH deficiency causes reduction in CR neuron numbers in the neocortex without enhanced apoptosis still remains an unresolved question.

BDNF being a TH-regulated gene that negatively regulates reelin (Ringstedt et al. 1998; Koibuchi et al. 1999) was suggested as a possible mediator of TH action on reelin (Alvarez-Dolado et al. 1999). BDNF and its receptor trkB are constitutively expressed throughout the migratory phase of E14–E18 under euthyroid state, but levels significantly change in the initial migratory stage of E14 under hypothyroidism (Fig. 4*A*,*B*). The changes in BDNF and its receptor trkB expression levels are paradoxical with persistent significant decrease in reelin expression under continued maternal TH deficiency given their inverse relationship. Therefore, contrary to earlier assumption, maternal TH does maintain the physiological levels of reelin essential for neuronal migration that seems to be independent of alterations in BDNF and its receptor levels.

We therefore looked into the possibility of direct TH action on *reelin* gene. TRs in the neocortex appear around E14 that coincides with reelin secretion (Bernal and Pekonen 1984; Bradley et al. 1992). The classical mechanism of action of TH is the regulation of gene transcription through the binding to specific nuclear receptors that interact with precise nucleotide sequences (TREs) present in target genes. We first confirmed the presence of TRα1 in the MZ of embryonic neocortex comprising mainly CR cells. Interestingly, increased TRα1 signals colocalized with reduced reelin under TH-deficient condition indicating a direct negative relationship between TRα1 and reelin expression. We next screened *reelin* gene for the presence of TRE. In silico prediction using NUBIScan and NHRScan softwares indicated the presence of a DR4 site residing in the first intronic region of *reelin*. Furthermore, our gel shift assay results validated these predictions. Moreover, we have also verified the physiological binding of TR on *reelin* gene by ChIP assay and identified it to be a target gene directly regulated during neocortical development. As depicted in Figure 5, only the DNA fragments immunoprecipitated by anti-TRα antibody are amplified by the reln1 primers that spanned the region containing TRE, while the amplification did not occur by reln2 primers that encompassed the region away from TRE; this proves the specificity of the TR interaction with *reelin* gene.

Effects of TH Deficiency on Reelin Signaling Cascade

Reelin binds to surface receptors ApoER2 and VLDLR (D'Arcangelo et al. 1999; Heisberger et al. 1999) on glial as well as neuronal cells and then induces tyrosil phosphorylation of an intracellular protein, Dab1 (Howell et al. 1999). Subsequently, ubiquitinated Dab1 degradation is necessary for correct layer formation in the forebrain (Feng and Cooper 2009). Our results show that both the lipoprotein receptor

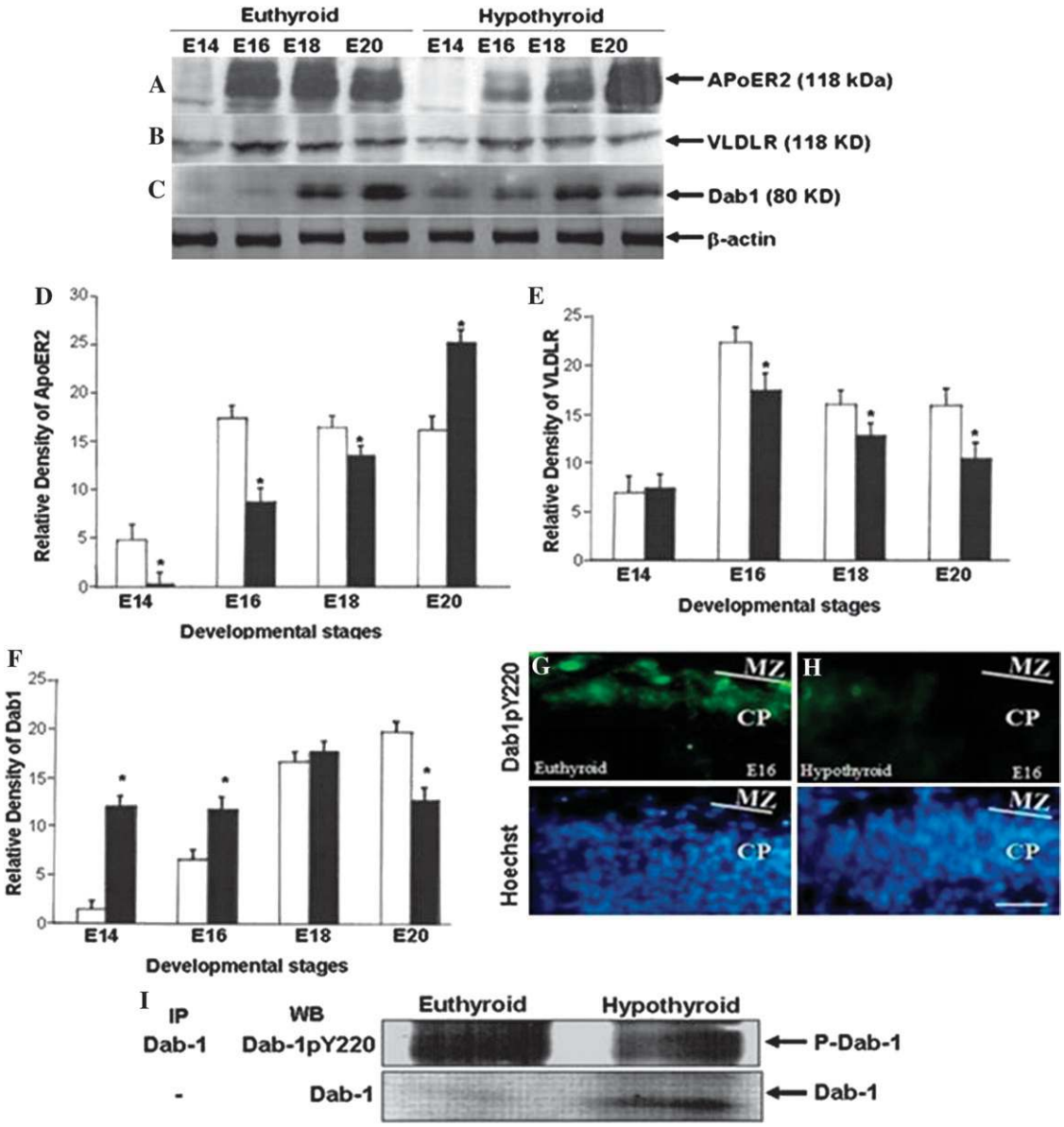

Figure 6. Diminished expression and activation of reelin receptors and Dab1 under TH deficiency. (*A–C*) Immunoblots of whole-cell lysate (100 μg) prepared from neocortices of the fetuses from E14 to E20, showing levels of ApoER2 (*A*), VLDLR (*B*), and Dab1 (*C*). (*D–F*) Relative density in euthyroid and hypothyroid groups. Each bar represents the mean of the respective individual levels ± standard error ($n = 5$ fetuses from different rats at each developmental stage). Significant differences compared with age-matched euthyroid counterpart are indicated ($^*P < 0.05$). (*G–I*) Phosphorylation status of Dab1 at specific tyrosine residue 220 in the cortical plate (CP). Immunofluorescence with tyrosine (220)–specific Dab1 antibody shows lesser number of positive cells (green) in the hypothyroid (*H*) CP in comparison with euthyroid (*G*) at E16. Nuclei were counterstained with Hoechst (blue). Immunoblot showing the reduced amount of phosphorylated Dab1 (Dab1 pY220, 80 kDa) and enhanced levels of total Dab1 content under TH deficiency as compared with euthyroid control at E16. Two hundred micrograms of protein from fetal neocortices was first immunoprecipitated with unphosphorylated Dab1 antibody, followed by immunoblotting with anti-Dab1 pY220 antibody (upper panel). The lower panel shows the western blot of abundance of unphosphorylated Dab1 in the fetal neocortex (scale bar *G*, *H*: 50 μm).

ApoER2 and VLDLR are significantly decreased at E16 although this downregulation appears to get compensated at later developmental stage under TH deficiency. These observations indicate that maternal TH deficiency exerts a compounding derangement of migration cues by repressing not only reelin levels but also that of its receptors. In contrast with the repression of reelin and its receptor, the levels of Dab1, however, registered an increase. The reduced phosphorylation of Dab1 under TH deficiency compared with euthyroid controls as seen at E16 helps to resolve this enigma. The loss of Dab1 activation is evident from its decreased phosphorylation at tyrosine residue 220, and increased levels of unphosphorylated Dab1 further reflect a compromised reelin signaling under maternal TH deficiency.

Reelin co-receptor $\alpha_3\beta_1$ integrin is necessary for neuron–glial recognition during neuronal migration, and α_v integrins provide optimal levels of the basic neuron–glial adhesion needed to maintain neuronal migration during development. β_1 integrin is required for glial anchorage to meningeal surface (Graus-porta et al. 2001). Under hypothyroid condition, the unaltered levels of α_3 integrin seen by us exclude the possibility of any recognition defect between radial glial scaffold and

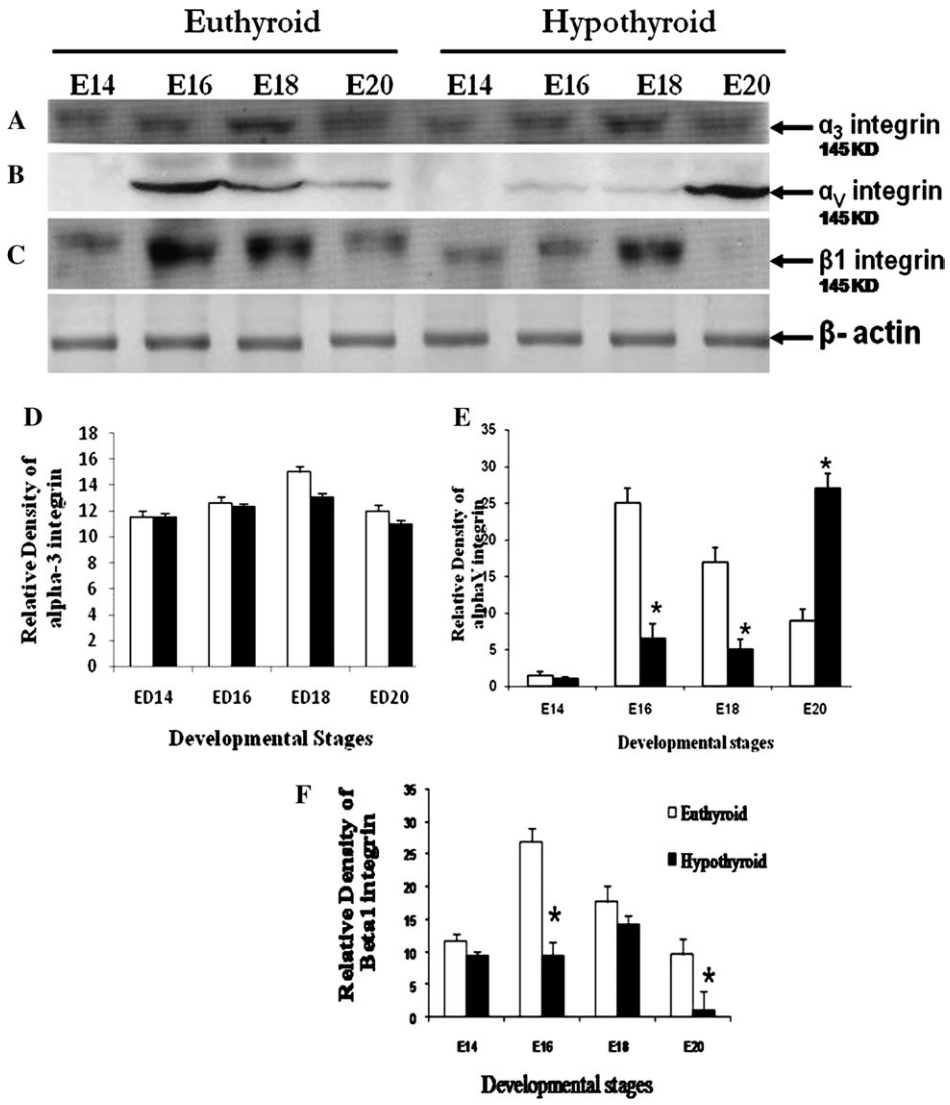

Figure 7. Effect of maternal hypothyroidism on integrin abundance in the neocortices from E14 to E20. Western blot analysis of whole-ell lysate (100 μg) revealed comparable levels of α_3 integrin (145 kDa) at all stages (*A, D*) under euthyroid and hypothyroid condition. Expression of α_v integrin (145 kDa) at E16 and E18 (*B, E*) and the levels of β_1 integrin (145 kDa) were reduced at all developmental stages (*C, F*) under hypothyroidism. (*D–F*) Relative density in euthyroid and hypothyroid groups. Each bar represents the mean of the respective individual levels. Results are expressed as means ± standard error of fetuses from 5 different dams in each group. Significant differences compared with age-matched euthyroid pups are indicated: $^*P < 0.05$.

migrating neurons. However, the decreased levels of α_v and β_1 integrins are suggestive of failure of neurons to maintain adhesion to glial scaffold resulting in their accumulation in lower layers V–VI. Altered expression of α_v and β_1 integrins in the embryonic cortex though indicate their possible role in radial glial cells' inability to provide necessary support for neuronal migration under hypothyroidism, this needs additional proving.

To conclude, data show that the direct TH action on *reelin* gene control is one of the molecular processes involved in TH-dependent neocortex development. First time demonstration of functional TRE in *reelin* gene, reduction in expression of lipoprotein receptors ApoER2 and VLDLR, as well as Dab1 phosphorylation and altered integrin levels under TH-deficient state are a step forward in providing mechanistic elucidation of the effect of TH-mediated synchronized migration of neurons during neocortex formation. Thus, maternal hypothyroidism–mediated cytoarchitecture defects including mispositioned neurons, disrupted barrel formation, blurring of different cortical layer borders, and loss of bipolarity of migrating neurons in the intermediate zone can be attributed at least in part to reelin signaling defects. The reversibility of neuronal positioning, neuroglial number, and morphology on early T4 treatment along with restoration of reelin levels in CR cells brings forth the importance of correction of maternal thyroid status early in pregnancy.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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