Transcriptional Repression of Neurotrophin Receptor trkB by Thyroid Hormone in the Developing Rat Brain*

Expression of the neurotrophin receptor trkB is regulated by thyroid hormone (T3) during development of the rat brain. trkB mRNA levels, coding for the full-length and the truncated isoforms, are increased in the cerebral cortex of neonatal experimental hypothyroid animals. Run-on transcription assays with nuclei from postnatal day 15, hypothyroid, and control cerebral cortices demonstrated that an increase in the transcription rate of the trkB gene accounts for the observed effect. Transient transfection experiments using a reporter plasmid containing a 7-kilobase pair DNA fragment upstream of the mouse trkB gene showed that unliganded thyroid hormone receptor (T3R) increases promoter activity, whereas addition of T3 reverses that activity below basal levels. Deletion analysis shows that the T3-dependent repression requires binding of the T3R to a specific region located downstream of the transcription start site. This region, at nucleotide position 465/–432, contains an array of thyroid hormone response half-sites that bind preferentially T3R as heterodimers with retinoid X receptor and whose deletion causes loss of the T3-dependent repression. These half-sites are able to confer negative regulation by T3 to a heterologous promoter, thus indicating the functionality of these sequences. These results demonstrate that, in the developing rat brain, T3 down-regulates the expression of the trkB gene through the active repression of a novel negative response element located downstream of its transcription initiation site.

Development of the nervous system follows a sequence of specific events, including proliferation of precursors, differentiation, survival of different cell types, and the establishment of the appropriate synaptic connections. Many of these processes require the coordinated action of intrinsic and extrinsic factors. Among the latter, hormones and neurotrophic factors regulate crucial events, such as differentiation and survival, which depend completely on the physiological availability of these molecules.

In mammals, the major hormonal regulator in the developing central nervous system is thyroid hormone (triiodothyronine, T3).1 Severe thyroid hormone deficiency in man leads to cretinism, a syndrome associated with mental retardation and neurological deficits (1). Animal models have showed that hypothyroidism during critical periods of development causes a vast array of abnormalities including incomplete maturation of neuronal and glial cells, reductions in the synaptic densities, myelin deficits, and changes in the number of specific cell populations (2). Most of the effects of T3 are mediated by binding and activation of specific high affinity nuclear receptors (T3Rs). Regulation of gene transcription by T3R involves hormone-dependent conformational changes in the T3R protein and sequence-specific interactions with thyroid hormone response elements (T3REs) in the target genes (for review, see Ref. 3). Unliganded T3Rs mediate transcriptional repression of positive T3REs because of binding of nuclear corepressors (4) that upon ligand binding are released and replaced by coactivators allowing transcriptional activation (5). The mechanisms involved in T3-dependent transcriptional repression remain less well defined. Negative response elements are located close to, and often downstream from, the transcriptional start site (6–9). T3Rs can bind to these sequences as monomers, homodimers, or heterodimers with retinoid X receptor (RXR) (6, 10, 11). In addition, T3R can also negatively affect the expression of certain genes, without requiring binding to DNA, by interfering with other transcription factors (12–14).

In contrast with the hormones, neurotrophic factors are provided locally and in very low amounts, controlling in this way the survival of the neuronal populations (15). Neurotrophins were first described in the peripheral nervous system, the best known being nerve growth factor. Other members of this family are brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 (16). Neurotrophins are recognized by high affinity tyrosine kinase receptors (Trk). TrkA is the receptor for nerve growth factor, TrkB for brain-derived neurotrophic factor and neurotrophin-4, and TrkC serves as the receptor for neu-

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1 The abbreviations used are: T3, triiodothyronine; T3R(s), thyroid hormone nuclear receptor(s); T3RE(s), thyroid hormone response element(s); RXR, retinoid X receptor; GADPH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; bp, base pair(s); kb, kilobase pair(s); EMSA, electrophoretic mobility shift assay; LUC, luciferase; N2a, neuro-2a; RSV, Rous sarcoma virus; GST, glutathione S-transferase; P, postnatal day.

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Thyroid Hormone Regulates trkB Expression

37511

rotrophin-3 (for review see Ref. 17). Alternative splicing within the trkB intracellular domain has been shown to generate isoforms with truncated or full kinase domain (18), which may have distinct signaling capabilities and cellular responses to the neurotrophins (19, 20). The pleiotropic action of T3 in brain development is probably mediated by the regulation of a few primary essential signals, and it has been suggested that neurotrophins and their receptors could be among them (21). Interestingly, we have previously shown that the ectopic T3Rα1 expression in a mouse neuroblastoma cell line induces the specific expression of trkB, with the concomitant repression of the other trk receptor genes (22).

In the present study, we investigated the effect of hypothyroidism on trkB expression in the developing brain and studied the molecular basis for such an effect. The results show that T3 deficiency induces an increase in the steady-state level of the trkB transcripts because of a higher rate of transcription of the trkB gene in hypothyroid animals. We find that the sequences required for the action of T3R as a ligand-dependent repressor are located downstream of the transcriptional initiation site. These sequences do not contain a consensus T3ERE but a series of imperfect widely spaced half-sites within the –437/–342 fragment, whose deletion causes loss of the T3-dependent repression. This sequence binds T3R/RXR heterodimers and confers repression by T3 to a heterologous promoter when it is inserted downstream of the transcription initiation site. These results indicate that the T3-dependent repression of trkB gene expression in the developing brain could be involved in the deleterious effects found in the neonatal hypothryoidism syndrome.

EXPERIMENTAL PROCEDURES

Animal Treatment

Wistar rats raised in the Instituto de Investigaciones Biomédicas animal facilities were used. European Union (D609) and Spanish (RD223/88) rules for maintenance and handling of laboratory animals were followed. To induce fetal and neonatal hypothryoidism, dams were given 0.02% methylmercaptoimidazol (Sigma) in their drinking water from the ninth day after conception. On postnatal day 5, pups were surgically thyroidectomized. This protocol resulted in profound hypothyroidism with dramatic decrease of T3 levels in the brain during the postnatal period (23). Animals were killed at different ages by decapitation, and brains quickly were removed, and the cerebral cortices were dissected on a chilled surface and frozen in liquid N2.

RNA Analysis

Preparation of RNA—Total RNA was obtained from different developmental ages from a pool of rat cerebral cortices by the method of Chomczynski and Sacchi (24).

Northern Blots—RNA (25 μg per lane) was denatured, electrophoresed through 1% agarose-formaldehyde gels, and transferred to nylon membranes following standard techniques. DNA probes for trkB (mouse trkB cDNA clone pFRK43; see Ref. 25), myelin-associated glycoprotein (clone 1B236–18 containing full-length rat cDNA; see Ref. 26), myelin basic protein (from Dr. A. Campagnoni), and cyclophilin (27) were labeled at high specific activity with32P-dCTP by multiprime. Hybridization was carried out overnight at 65 °C according to Church and Gilbert (28). Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer, pH 7.2, at 65 °C, and membranes were exposed to x-ray film at –70 °C with intensifying screens.

RNase Protection Assays—32P-labeled antisense riboprobes were prepared by in vitro transcription with T3 or T7 RNA polymerase (Promega) in the presence of 32P-dCTP. Antisense cRNA probe for full-length and truncated rat trkB were prepared as described (29). In RNase protection assay, the probe gives two protected fragments of 390 and 238 bp that correspond, respectively, to the transcripts encoding the kinase and the truncated versions of the TrkB receptor. As an internal control, an antisense cRNA probe detecting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was synthesized using a fragment spanning from nucleotides 196 to 357 of the rat GAPDH cDNA (29). RNA protection assays were performed using the RPAII Ribonuclease Protection Assay kit (Ambion). In each reaction, 20 μg of total RNA was hybridized to the antisense trkB and GAPDH cRNA probes as recommended by the manufacturer. Protected fragments were separated on 6% polyacrylamide sequencing gels, and the gels were dried and exposed to x-ray films at –70 °C with intensifying screens. Quantification of the radioactivity present in the protected bands was made with an Instant Imager (Packard Instruments). Values obtained for trkB-protected fragments were corrected with the values obtained for GAPDH-protected fragments.

Run-on Assay

To measure the transcriptional activity of the trkB gene we isolated nuclei from cerebral cortices of euthyroid and hypothyroid 15-day-old rats. Brains from cortices were homogenized in 0.25 mM sucrose, 10 mM HEPES, pH 7.4, containing 0.5% Nonidet P-40 (Sigma) using a Dounce homogenizer at 4 °C. Nuclei were washed once in the same buffer and twice in the same buffer without Nonidet P-40. Nuclei storage, in vitro run-on transcription assays, and hybridization conditions were carried out as described (23) except that the 32P-labeled RNA transcripts were hybridized to nylon filters containing an antisense trkB riboprobe. As control we used either the sense trkB riboprobe, as well as the neural cell adhesion molecule and cyclophilin cDNAs.

Transient Transfections

Plasmids—A 7-kb fragment from the 5’-flanking region of the mouse trkB gene (from plasmid pS3S4, a gift from Dr. M. Barbacid, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain), was subcloned into a derivative of the promoterless pBlCAT-3 plasmid lacking the AP-1-binding site present in the pUC backbone. This construct, spanning from –7059 (Sal I) to –325 (Cla I) with respect to mouse trkB translation start (–7059.CAT) was used to generate the following series of 5’-deletion mutants by means of convenient restriction sites as depicted in Fig. 3: –5130.CAT, –2698.CAT, –1606.CAT, and –871.CAT. For the analysis of the T3-dependent trkB repression, a series of constructs were derived from –871.CAT that included different fragments generated by polymerase chain reaction. 5’-deletion generated constructs –711.CAT, –632.CAT, and –465.CAT. Further 3’-deletion of those sequences generated construct –711/–432.CAT. The reporter plasmid pTL- (–437/–342)-LCUC consists of a single copy of the sequence comprised within the –437/–342 fragment and subcloned into the HindIII site located 3’ of the basal promoter in the reporter plasmid pTL-LUC (30). The pSG-5-derived expression vectors for chick thyroid hormone receptor α1, human thyroid hormone receptor β1, the c-erbB/ c-erba chimera pSG-C7, and N-terminal deletion mutant pSG-(His)7-DE-cTrc1 have been previously described (31). Retinoic expression vectors encoding the human α, β, γ or retinoic acid receptor isoforms cloned in the pSG-5 plasmid and were supplied by Dr. H. Stunnenberg (University of Nijmegen).

Cell Culture and Transfection Experiments—Neuro-2A (N2a) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were plated at a density of 120,000 cells per 3-cm dish on the day before transfection. Before transfection, medium was removed, and transfection was performed by the calcium phosphate method. Each 3-cm dish received 2 μg of the appropriate reporter construct, 1 μg of the internal control plasmid pRSV-LacZ, and up to 2 μg of the expression vector encoding T3Rs or empty pSG-5 vector. 16–18 h after DNA addition the cells were washed with PBS and incubated with medium containing serum depleted of T3 and retinoids (when required) by treatment with charcoal and Dowex resin. Where appropriate, T3 (Sigma) or all-trans-retinoic acid (Sigma) were added at 150 nM and 1 μM, respectively, and the cells were incubated for another 24 h before harvesting for determination of β-galactosidase and luciferase or CAT activities.

Electrophoretic Mobility Shift Assay (EMSA)

For EMSA we used purified recombinant TRα1 and RXRα expressed in Escherichia coli as GST fusion proteins (a gift from Dr. A. Aranda, Instituto de Investigaciones Biomédicas, Madrid, Spain). Labeled DNA fragment (–466/–320) was generated in a polymerase chain reaction reaction with specific primers, in which one of the primers was previously labeled to high specific activity with [32P]γ-ATP and T4 polynucleotide kinase. Protein extracts were incubated with this labeled DNA fragment in 18 μl of binding buffer (15% glycerol, 5 mM MgCl2, 50 mM KCl, 20 mM HEPES, pH 7.5, 5 μM diothiofritol, 5 μg/ml bovine serum albumin (Sigma; catalog number B8667), 0.1% Triton X-100, and 0.3 mg/ml poly(dI-dC) for 15 min on ice. 40–100 fmol of labeled probe was
Hypothyroidism Increases Steady-state Levels of the trkB mRNAs and trkB Transcription Rate in the Postnatal Cerebral Cortex—The trkB gene encodes two receptor isoforms that are generated by differential splicing. One corresponds to the full-length isoform with kinase activity, the other to the truncated isoform. Their expression starts very early during brain development and is widely distributed among different brain regions.

**RESULTS**

Hypothyroidism Increases Steady-state Levels of the trkB mRNAs and trkB Transcription Rate in the Postnatal Cerebral Cortex—The trkB gene encodes two receptor isoforms that are generated by differential splicing. One corresponds to the full-length isoform with kinase activity, the other to the truncated isoform. Their expression starts very early during brain development and is widely distributed among different brain regions.
by hypothyroidism, and also for a neural cell adhesion molecule that is transcriptionally repressed by T3 during brain development (34). As control for nonspecific hybridization, the trkB riboprobe in the sense orientation was used. The relative rates of trkB gene transcription resulted in a 1.9-fold increase in the hypothyroid versus the euthyroid animals. These data provide clear evidence for a direct effect of T3 on the transcription rate of the trkB gene in vivo.

**T3R-mediated Repression of the trkB Promoter Activity by T3**—Transient transfection assays were carried out to confirm that T3 represses the transcriptional activity of the trkB gene. We used a reporter plasmid containing a 6740-bp fragment from the 5'-flanking region of the trkB linked to the CAT gene. This fragment, spanning from −7059 to −325 with respect to the ATG translation initiation codon, has been completely sequenced. It contains two alternative promoters, whose transcription initiation sites are located at −1800 (promoter P1) and −448 (promoter P2) (35). As shown in Fig. 3, cotransfection of this −7059.CAT reporter plasmid with different vectors expressing either α or β T3R isoforms produced a dramatic increase in the CAT activity. Addition of T3 effectively reversed this effect. Thus, T3-dependent repression and the unliganded T3R activation did not exhibit T3R isoform specificity. In contrast to the previously reported stimulation of trkB expression in human neuroblastoma cell lines by retinoic acid (36), we did not observe activation of the −7059.CAT after transfection with different retinoic acid receptor isoforms and addition of its ligand (Fig. 3).

**Identification of the DNA Region Responsible for the T3-dependent trkB Repression**—To obtain further information on the cis-acting elements involved in the regulation of the trkB gene by T3R, we studied the activity of different deletion constructs after cotransfection with the T3Rα1 expression vector and addition of T3. As shown in Fig. 4, T3Rα1 increased the promoter activity in every construct, and this increase was higher in the constructs that contained sequences between −5130 and −2698. T3 repressed the promoter activity below basal levels (−75% inhibition of maximal activity) in every construct. No differences were found between the constructs that contained...
FIG. 5. T3-dependent repression of the trkB gene is mediated by elements located downstream the transcription start site.

The effect of T3 on the transcriptional activity of deletion constructs from the region −871/−325 was studied in transient transfection experiments in N2a cells. A, sequence of the −871/−325 region, B, a representation of the analyzed region is depicted. The relevant sequence features are shown schematically, and the positions of the end points of the 5′- and/or 3′-deletion reporter constructs are indicated. C, transient transfection with different deletion constructs from the region −871/−325 were performed in N2a cells. Each 30-mm cell dish received 2 μg of the CAT reporter and 1 μg of the internal control plasmid RSV-LacZ, with 2 μg of pSG-5-derived expression vector for chick T3Rα (hatched and black bars) or empty vector (white bars). After transfection the medium was replenished, solvent (white and hatched bars) or 150 nM T3 (black bars) was added, and cells were further processed as indicated in the legend of Fig. 2. CAT values were corrected for transfection efficiency with their respective β-galactosidase activity values and were expressed as relative CAT activity in arbitrary units. Data for the −871.CAT reporter are the mean ± S.D. from four different experiments performed in duplicate, and data for the −465.CAT reporter construct are the mean of duplicate independent experiments.

To identify the sequences required for the T3-dependent repression, we studied the activities of new 5′-deletion constructs generated from the −871.CAT plasmid (Fig. 5). All 5′-deletion constructs were repressed more than 60% by T3. The negative effect of T3 was only lost in the 3′-deletion construct −711/−432.CAT. These results suggested that the T3-dependent repression sequence must be located downstream of the transcription initiation site within the sequence −432/−325.

FIG. 6. T3R requires the AF-2 and DNA binding domains to mediate the T3-dependent repression of trkB promoter. A, schematic representation of the T3R mutants used for the experiment. The DNA binding domain is shown as a stippled box. Sequences derived from T3Rα1 are shown filled, whereas the regions derived from v-erbA, the oncogenic variant of T3Rα1, are shown as an empty box in which dots are the mutated residues. B, effect of different T3Rα1-derived mutants on the transcriptional activity of trkB reporter constructs −871.CAT and −465.CAT. Transient transfections were performed in 30-mm N2a cell dishes with 2 μg of the CAT reporter and 1 μg of the RSV-LacZ internal control plasmid. 2 μg of pSG-5-derived expression vector for chick T3Rα1 or the corresponding T3Rα1-derived chimeras as indicated (black and gray bars) or empty vector (white bar) were co-transfected. 18 h later, the medium was replenished, and solvent (white and hatched bars) or 150 nM T3 (black bars) was added. The cells were further processed as indicated in the legend of Fig. 2. CAT activity values were corrected for transfection efficiency with their respective β-galactosidase activity values and were expressed as relative CAT activity in arbitrary units. Data for the −871.CAT reporter are the mean ± S.D. from four different experiments performed in duplicate, and data for the −465.CAT reporter construct are the mean of duplicate independent experiments.

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activity of −871.CAT and −465.CAT reporter constructs after cotransfection with several T3Rα1 mutants (Fig. 6). We found that neither the cT3Rα-C1 receptor mutant, which carries a 9-amino acid C-terminal deletion in the AF-2 domain (31) nor the N-terminal deletion mutant, (His)6-DE-cT3Rα, which lacks the DNA binding domain, were able to mediate repression by T3 (Fig. 6). In contrast, the cT3Rα-C7 receptor mutant (a chimera between cT3Rα1 and its oncogenic variant v-erbAα), in which dimerization with RXR is severely impaired when RXR concentration is limiting (37), has the same repressor effect as the wild-type receptor (Fig. 6).

Identification of the TR Binding Element in the −465 to −325 Region of the trkB Gene—To characterize the DNA sequence recognized by the T3Rs we performed DNase protection experiments using recombinant T3Rα1/RXRα heterodimers and the DNA fragment spanning from −711 to −339. We found a partly protected region flanked by hypersensitive sites between nucleotides −441/−345 (data not shown). This region included four imperfect T3RE half-sites immediately 3′ to the transcription initiation site (see Fig. 5A). Accordingly, the fragment between −466 and −339 was able to bind heterodimers of T3Rα1/RXRα in EMSA assay. As is shown in Fig. 7, one major specific binding complex was observed that could be competed with the unlabeled fragment or a consensus T3RE oligonucleotide. A weak binding of T3Rα1 could also be observed under certain conditions, but the binding was greatly improved by the addition of RXRα (Fig. 7A). The presence of T3 did not modify the extent of specific binding of this fragment with the heterodimers T3Rα1/RXRα (Fig. 7B). Hence, the T3-dependent repression of the trkB gene appeared to be mediated by the interaction of T3Rα1/RXRα heterodimers with four imperfect half-sites (nT3-responsive sequence) located immediately downstream of the transcription initiation site between positions −440/−347.

The Novel Negative T3-responsive Sequence of the trkB Gene Confers T3-dependent Repression to a Heterologous Promoter—To confirm the functionality of the sequence characterized as the one that mediates the T3 repression of the trkB gene, we studied whether this sequence was able to confer T3 responsiveness to a heterologous neutral promoter. For this aim, N2a cells were transiently cotransfected with a reporter plasmid that contains the −437/−342 bp fragment of the mouse trkB gene linked 3′ into the basal reporter pTI-LUC (30) and the T3Rα1 expression vector. As shown in Fig. 8, the activity of this promoter was significantly increased by the unliganded T3R, and T3 reversed this effect to the basal levels.

as control, the activity of the reporter plasmid pTI-LUC was unaffected by either the expression of the T3R or the addition of T3. Other reporter plasmids generated by the insertion of several oligonucleotides spanning combinations of two of the various half-sites (i.e. half-sites 1 and 2, or 2 and 3, or 3 and 4) into the plasmid pTI-LUC were used to study the contribution of the different half-sites to T3-repression activity. Interestingly the activity of these constructs was not affected by T3R.
either in the presence or in the absence of T3 (results not shown), suggesting that the four imperfect half-sites are able to generate a novel negative T3RE.

**DISCUSSION**

The data presented in this study show that trkB expression is regulated by T3 during neonatal brain development. Expression of both receptor isoforms (truncated and full-length) is regulated in the same fashion. As with other T3-regulated genes (33), the observed effect depends on the brain region. We find an increase in trkB mRNAs in cerebral cortex and subcortical structures of hypothyroid animals. This effect persists throughout the postnatal period in the cortex but not in the subcortical structures, where it normalizes after three weeks. Previously, a report studying only cerebellum found no significant changes in this structure (29). The cause(s) of this regional difference in the action of T3 is unknown, and it is likely that other developmental and spatial-specific factors might be involved.

The increased trkB expression found in the neonatal hypothyroidism is due to an increased transcriptional rate, indicating that T3 suppresses the transcription of this gene in vivo. Indeed, gene repression may play an important role in the regulation of brain development by T3, because many of the new T3-regulated genes are also transcriptionally repressed in brain (34, 38, 39). Moreover, it has been suggested that neurotrophins and their receptors could mediate some of the known functions of T3 during the central nervous system development (21). For instance, hypothyroidism disrupts the developmental pattern of the four neurotrophins in cerebellum (29). However, the regulation trkB expression by T3, reported here, represents the first example where a direct transcriptional regulation of the neurotrophin and neurotrophin receptor gene families has been shown.

In contrast to a large number of relatively well characterized positive T3REs, much less is known on the few described that mediate T3-dependent repression. In general, unliganded receptor increases the transcriptional activity of these negatively regulated genes, and T3 reverses this effect below basal levels. We find that the trkB gene follows this pattern. Thus, the CAT reporter construct containing 7 kb of 5'-sequence of the trkB gene shows an enhanced promoter activity when T3 expression vectors are cotransfected, whereas T3 addition reverses this activation and causes repression below the original basal level.

In this paper we demonstrate, using transient transfection experiments with various 5'- and 3'-deletion constructs of the trkB gene (Figs. 4 and 5), that the T3-responsive sequence is located in a region 3' from the transcription initiation site of the trkB gene. It is interesting to note that the T3RE location downstream of the transcriptional start site has also been reported in other T3 negatively regulated genes (11, 38, 40, 41). The mechanism by which T3R represses transcription in a strictly ligand-dependent manner has been elusive. We find that the repression of trkB by T3 requires its DNA binding domain of T3R (Fig. 6). Interestingly, in the thyroid-stimulating hormone β promoter, it has been described that T3R interacts through its DNA binding domain with histone deacetylase 2. The addition of T3 enhances the recruitment of T5R and histone deacetylase 2 to its negative T3RE (9). Besides, the AF-2 is also implicated in the T3-dependent regulation of the trkB gene (Fig. 6). This region appears to be mainly involved in the binding of coactivators (42), suggesting that these proteins might also play a role in the regulation of this gene by T3.

Finally, the trkB region found to be responsible of the T3-dependent repression comprises several imperfect T3RE half-sites widely spaced. This region is able to bind heterodimers T3Ra1/RXRα in an EMSA (Fig. 7) and confers negative regulation to a heterologous promoter, confirming the functionality of this sequence in T3R binding (Fig. 8). The length of this sequence opens the possibility that it may harbor a composite site involving the receptor and other factors as it has been described in other genes (43, 44) and whose nature will be worth exploring in the future.

In summary, this report demonstrates the negative regulation of the trkB gene by T3 in the developing brain. Studies on mice with null mutations for the trkB receptor have highlighted many actions that require the expression of this receptor during peripheral and central nervous system development (45). Thus, alteration of the proliferation/differentiation and survival of defined neoblast populations by the deregulation of trkB expression in the hypothyroid neonatal brain might explain some of the deleterious effects found in this syndrome.

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