MOLECULAR AND CELLULAR BIOLOGY, June 2006, p. 4311–4315 Vol. 26, No. 11
0270-7306/06/$08.00+0 doi:10.1128/MCB.02158-05

Congenital Hypothyroidism (Cretinism) in neuroD2-Deficient Mice

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Received 7 November 2005/Returned for modification 9 December 2005/Accepted 9 March 2006

Mice lacking neuroD2, a basic helix-loop-helix transcription factor involved in brain development, show growth retardation and other abnormalities consistent with hypothalamic-pituitary-thyroid (HPT) axis dysfunction. neuroD2 is expressed in the paraventricular hypothalamic nuclei, the anterior lobe of pituitary, and the thyroid gland. In neuroD2-deficient mice, thyrotropin-releasing hormone, thyroid-stimulating hormone, and thyroid hormone are decreased in these three regions, respectively. neuroD2-null mice typically die 2 to 3 weeks after birth, but those treated with replacement doses of thyroxine survived more than 8 weeks. These data indicate that neuroD2 is expressed throughout the HPT axis and that all levels of the axis are functionally affected by its absence in mice.

Proper development of brain and other tissues in the neonatal and infant periods depends on adequate thyroid hormone levels. Congenital hypothyroidism in newborns occurs in one in 4,000 births, making it the most common hormonal disorder in infants (4, 22). Neonatal hypothyroidism may be caused by thyroid gland dysgenesis (primary hypothyroidism) due to either ectopia or hypoplasia/aplasia of the gland or by insufficiency of the hypothalamic cells (thyrotropin-releasing hormone [TRH] deficiency) or pituitary cells that secrete thyrotropin (thyroid-stimulating hormone [TSH]) (secondary hypothyroidism) (15, 17, 19). If undetected, neonatal hypothyroidism leads to severe mental and growth retardation, a syndrome known as cretinism. The cause of congenital hypothyroidism is typically unknown in most cases.

The paraventricular hypothalamus secrets TRH, which modulates the secretion and synthesis of TSH (thyrotropin) in the anterior pituitary through transcriptional activation of the TSH promoter (7, 24). TSH is transported in the bloodstream to the thyroid gland, where it positively regulates thyroglobulin, the precursor of thyroxine. Thyroxine binds to thyroid hormone receptors to control basal metabolic rate, growth, and maturation and affects almost every organ in the body (6, 11). Thyroxine also negatively regulates TRH. Very little is known about transcription factors that positively regulate the hypothalamic-pituitary-thyroid (HPT) axis.

Basic-helix-loop-helix (bHLH) transcription factors are involved in cell fate determination and differentiation in a variety of cell types during development. Studies with *Xenopus*, *Drosophila*, and mice have demonstrated that bHLH proteins are involved in developmental events such as cellular differentiation, lineage commitment, neurogenesis, myogenesis, hematopoiesis, pancreatic development, and sex determination (1–3, 5, 8).

Members of the neuroD subset of bHLH transcription factors were first characterized as neuronal differentiation genes because they are sufficient to induce cell cycle arrest and contribute to neuronal maturation (5, 12, 16). In addition, neuroD has an established neuroendocrine role in regulation of insulin secretion from pancreatic β cells and transcriptional regulation of proopiomelanocortin in pituitary (9, 21). The role of neuroD2 in the regulation of neuroendocrine function has not been previously established.

Here we evaluated neuroD2 expression in the pituitary gland and thyroid in addition to the previously described expression in paraventricular hypothalamic nuclei (PVN) (14). We measured TRH and TSH mRNA and serum thyroxine levels. In response to findings that the entire HPT axis is affected by the absence of neuroD2, we treated neuroD2-null mice with thyroxine to determine whether this rescues portions of the neuroD2-null mouse phenotype.

MATERIALS AND METHODS

Quantitative RT-PCR. Mice were euthanized under an IACUC-approved protocol, and pituitary glands and thyroid tissues were isolated for RNA extraction with Trizol reagent (Invitrogen) and converted to cDNA by using an ABI Tagman reverse transcription kit. Seven hormones expressed in anterior lobe of pituitary were analyzed by real-time quantitative reverse transcription-PCR (RT-PCR) performed on an ABI prism 7000 with SYBR green PCR master mix. Each sample was run in triplicate and normalized to mouse S16 internal control. SYBR green dye intensity was analyzed with SDS software. Five sets of mice were analyzed by real-time quantitative RT-PCR. The TSH level was further confirmed by [α-^{32}P]dCTP-radiolabeled RT-PCR from another three sets of pituitary samples. Specific primers for S16 (AGGAGCGATTTGCTGGTGT GGA and GCTACCAGGCCTTTGAGATGGA), follicle-stimulating hormone (FSH) (GATCTGGTGTATAAGGACCC and CCTTCATTTCACTGAAGG AGC), adrenocorticotrophin hormone (CCTACTCCATGGAGCACTTC and GAACTCTAGGGGAAAGGCC), prolactin (CTGCCAATCTGTTCCGCTG and GAGGGACTTTCAGGGCTTG), growth hormone (GH) (GGAGCTG GAAGATGGCAGC and CTAGAAGGCACAGCTGCTTTC), luteinizing hormone (LH) (GCTGCTGAGCCCAAGTGTG and GCAAGCTCCCGGTAG GTG), TSH-α (CACCTGCCCAGACACATC and GCGCTCAGAAGCTAC GAC), TSH-β (GTCTGTACATACAGAGACTTC and GACTGCGGCTTG GTGCAG), and thyroglobulin (GAGCTCAGTCTGCCTGG and GTGAC CTGTCTCCTACTC) were used.

RNA in situ hybridization, immunostaining, and histology. Mice were anesthetized and euthanized under an IACUC-approved protocol and then perfused with phosphate-buffered saline and fixed with 4% paraformaldehyde. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed as described previously (14). Tissues were Parafilm embedded and processed into

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12-μm sections after X-Gal staining. Fast red (Vector) was used for counterstaining. The 12-μm frozen brain sections were cut with a cryostat for RNA in situ hybridization and immunostaining. The TRH construct was amplified from a cDNA library and cloned into the pCDNA3 vector. A digoxigenin-labeled RNA probe of TRH was made with a digoxigenin RNA labeling kit (Roche) according to the manufacturer's instructions. Double labeling with anti-TSH-β (Chemicon; 1:500, rabbit polyclonal)) and β-galactosidase (Promega; 1:1,000, mouse monoclonal) was performed on 12-μm Parafilm-embedded pituitary sections. Alexa Fluor 594- and 488-conjugated secondary antibodies (Molecular Probes; 1:500), respectively, were used for detection. Immunostaining was performed on 12-μm frozen thyroid/parathyroid sections with antibodies against neuroD2 (1:500 dilution; generated in the lab of S. J. Tapscott), followed by secondary biotinylated rabbit immunoglobulin G (Vector; 1:200 dilution). ABC Elite and a diaminobenzidine substrate kit (Vector) were used for signal amplification and detection according to the manufacturer's instructions. Hematoxylin was used for counterstaining. Hematoxylin-eosin staining was performed on 12-μm frozen thyroid/parathyroid sections for thyrocyte quantification.

Northern blot analysis. One milliliter of Trizol reagent was used to extract total RNA from forebrain, 10 pituitary tissues, and 4 thyroid/parathyroid tissues. Electrophoresis for total RNA was performed on a 1% agarose gel containing 2.2 M formaldehyde, followed by transfer to nitrocellulose membrane and prehybridization. The neuroD2 cDNA probe was added for hybridization. The probe was synthesized and radiolabeled with [α-^{32}P]dCTP by using a random priming DNA labeling kit (Roche). The product was exposed and scanned with a phosphorimager.

Determination of thyroxine, growth hormone, and IGF2 levels. Mice were euthanatized according to an approved protocol. Blood was obtained from the renal vein and collected into EDTA-coated tubes. Serum was isolated after centrifugation and sent to Phoenix Central Laboratory (Everett, Washington) for thyroxine, growth hormone, and insulin-like growth factor 2 (IGF2) detection.

RESULTS

neuroD2-null mice experienced progressive neurological deterioration, growth retardation, and early death at postnatal day 2 (P2) to P21 (20). Because these components of the phenotype are consistent with cretinism, we systematically evaluated the HPT axis to determine the extent to which the absence of neuroD2 affected the molecules that regulate thyroid function.

Hypothalamus. In neuroD2 mutant mice, the coding region of neuroD2 is replaced by the LacZ gene, allowing the detection of neuroD2 expression by beta-galactosidase (X-Gal) staining. We previously reported that the neuroD2 gene locus was active in hypothalamic periventricular nucleus neurons. Beta-galactosidase staining of PVN revealed that the neuroD2 locus was active in both neuroD2-heterozygous and -null animals (Fig. 1A and B), demonstrating that neuroD2-expressing neurons in the PVN are present in neuroD2-null mice.

Several populations of hypothalamic neurons produce TRH, but previous reports indicated that only those that reside in PVN control TSH secretion (18, 25). To determine whether TRH levels were affected in neuroD2-null mice, the expression of TRH in PVN was analyzed by RNA in situ hybridization (Fig. 1C and D). TRH expression was absent in neuroD2-null animals (Fig. 1D), suggesting that neuroD2 regulates TRH transcription. Under lower magnification of the in situ analysis (Fig. 1E and F), TRH-positive signals were present in most areas of the hypothalamus but absent in PVN, where neuroD2 is expressed. This result suggests that neuroD2 specifically regulates TRH expression in the PVN cell lineage and that TRH is regulated by other transcription factors elsewhere in the hypothalamus. Because the TRH-positive PVN neurons regulate TSH, we next evaluated the pituitary.

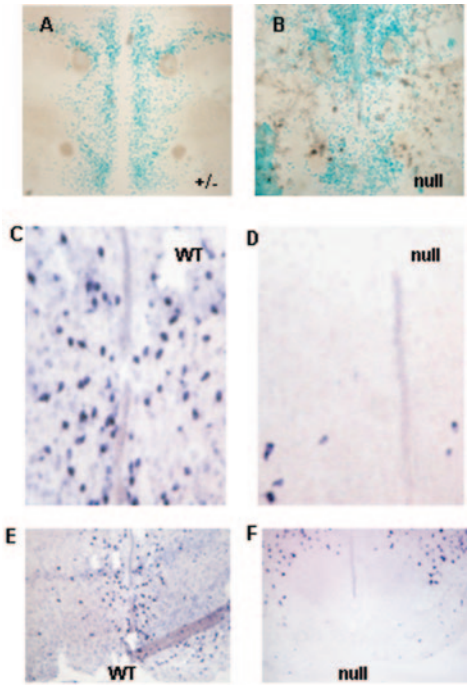

FIG. 1. neuroD2 is expressed in PVN and regulates TRH expression specifically in PVN. (A and B) neuroD2 expression in PVN was detected by X-Gal staining. The positive β-galactosidase activity was also detectable in PVN of neuroD2-null mice, suggesting that neuroD2-expressing cells are not absent in PVN. (C and D) Detection of TRH expression in paraventricular hypothalamic nuclei by RNA in situ hybridization. TRH is expressed in paraventricular hypothalamic nuclei of wild-type (WT) and neuroD2-heterozygous mice. TRH expression in paraventricular hypothalamus is diminished in neuroD2-null mice. Magnification, ×20. (E and F) Lower magnification (×4) shows that TRH expression is affected only in PVN and not in other hypothalamus area.

Pituitary. Beta-galactosidase staining of whole pituitary revealed that neuroD2 is expressed in this gland (Fig. 2A and B). The staining pattern in whole pituitary suggested that the neuroD2 locus was expressed primarily in the anterior lobe. This was confirmed on sections of pituitary, which showed activity in the anterior lobe, but not the posterior or intermediate lobe, of both neuroD2 heterozygotes and null mice (Fig. 2C and D). Immunostaining with anti-β-galactosidase antibody confirmed that neuroD2 is expressed only in anterior lobe of pituitary gland (Fig. 2E).

Hormones produced by the anterior part of the pituitary gland include adrenocorticotropic hormone (ACTH), FSH, LH, GH, prolactin, and TSH (23). To determine whether the levels of mRNAs that encode these hormones are affected in neuroD2-null pituitary gland, five sets of pituitary samples from wild-type and null littermates were analyzed by real-time quantitative RT-PCR with SYBR green fluorescence. Normalization was done with endogenous S16 and β-actin genes. The results showed that TSH-β was consistently decreased in five pituitary samples from neuroD2-null mice (Fig. 3A). The levels of ACTH, FSH, LH, GH, and TSH-α were not consistently elevated or decreased in neuroD2-null mice (Fig. 3A). TSH is a heterodimer of two unique proteins encoded by two different genes, α and β. An additional three sets of pituitary samples

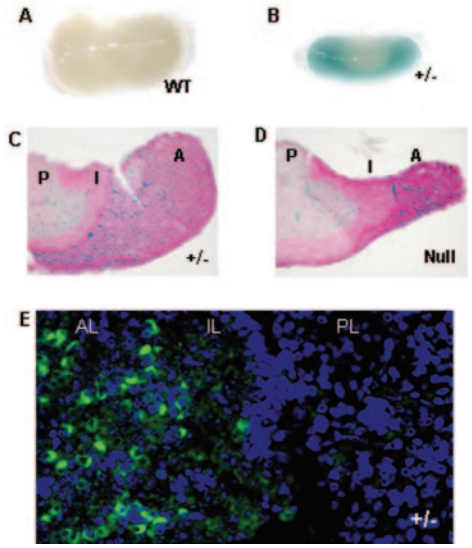

FIG. 2. neuroD2 is expressed in anterior pituitary. (A and B) X-Gal staining demonstrated neuroD2 expression in pituitary. (A) Wild-type (WT) pituitary as negative control; (B) heterozygote pituitary showed positive β-galactosidase activity. (C) neuroD2 is expressed abundantly in the anterior lobe of pituitary gland by X-Gal staining (20× amplification). Blue signal represents β-galactosidase activity in neuroD2-heterozygous mice. (D) β-Galactosidase activity is detectable in neuroD2-null pituitary gland (20× amplification), suggesting that neuroD2-expressing cells are not absent. (E) Immunostaining with anti-β-galactosidase antibody confirmed that neuroD2 is expressed only in anterior lobe. A, anterior lobe; I, intermediate lobe; P, posterior lobe.

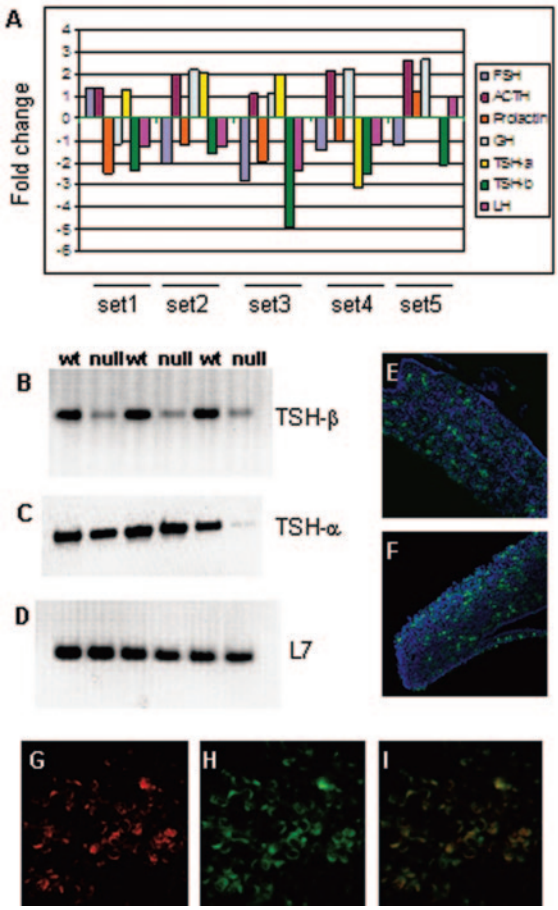

FIG. 3. Detection of the transcription levels of hormones produced from anterior pituitary gland. (A) Real-time RT-PCR detected the RNA levels of hormones expressed in the anterior pituitary glands from five sets of littermates, with normalization to S16. Each bar with a different color represents the fold change of different hormones compared to that in the wild-type littermate. The TSH-β level is consistently decreased in neuroD2-null mice. (B to D) The TSH-β level determined by radiolabeled RT-PCR was decreased in neuroD2-null mice (B), but TSH-α levels were similar in neuroD2-null mice and controls (C). Ribosomal protein L7 was used for internal control (D). wt, wild type. (E and F) Immunostaining with two different TSH-β antibodies reveals specific detection of TSH-β in anterior pituitary. (G to I) Double labeling of TSH-β and β-galactosidase in neuroD2-heterozygous pituitary sections reveals that neuroD2 is colocalized with TSH-β. (G) TSH-β immunostaining; (H) β-galactosidase immunostaining; (I) merged image of TSH-β and β-galactosidase immunostaining.

from wild-type and null littermates were analyzed by [α-^{32}P]dCTP-radiolabeled RT-PCR with the internal control of ribosomal protein L-7 (Fig. 3D). In these three sets of pituitary samples, TSH-β expression was consistently decreased in null mice (Fig. 3B). In contrast, the TSH-α expression level analyzed by radiolabeled RT-PCR and real-time RT-PCR was not consistently affected in neuroD2-null compared to wild-type mice (Fig. 3C).

To determine whether TSH is normally expressed in neuroD2-positive neurons in the anterior pituitary, we conducted colocalization studies. We first compared two different TSH antibodies and found that both specifically bound to neurons in the anterior pituitary (Fig. 3E and F). We then costained sections of pituitary from a neuroD2 heterozygote with one of these antibodies and an antibody that recognizes beta-galactosidase. This revealed that TSH is produced in neuroD2-positive neurons of the anterior pituitary but not in neuroD2-negative neurons (Fig. 3G, H, and I). It was not possible to measure TSH protein levels in serum because the amount of serum required (1 ml) far exceeds the amount that could be obtained from these growth-arrested mice.

Thyroid. To complete our analysis of the HPT axis, we evaluated neuroD2 expression in the thyroid gland. neuroD2 was detected in thyroid tissue by Northern blot analysis (Fig. 4A). The thyroid gland contains follicles filled with eosinophilic colloid and lined by epithelial cells. The products of follicular cells are T3 and T4 (thyroxine and thyroid hormone). Calcitonin-producing parafollicular C cells are located between the thyroid follicles (Fig. 4B). The number of thyrocytes was evaluated and counted after hematoxylin-eosin staining, and there was no significant difference between wild-type and null mice (wild type, 143 ± 23; null, 135 ± 20). To distinguish which cell types express neuroD2, immunostaining with neuroD2 antibody was conducted on serial sections of thyroid and parathyroid glands. neuroD2 was expressed primarily in thyrocytes, although some expression was noted in small populations of parafollicular C cells (Fig. 4C). X-Gal staining on neuroD2-heterozygote and -null thyroid glands confirmed that neuroD2-expressing cells were present in neuroD2-null mice (Fig. 4D and E).

Thyroglobulin, a precursor of thyroid hormone, is stored in

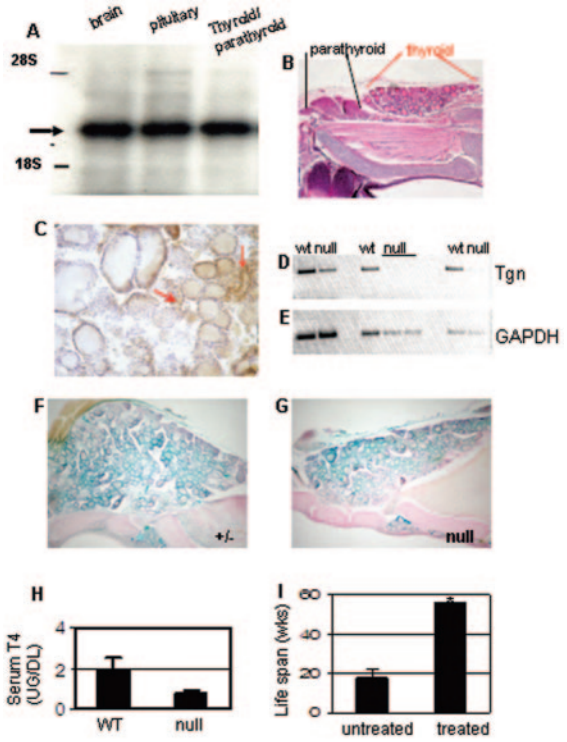

FIG. 4. neuroD2 is expressed in thyroid gland and affects serum T4 level, and thyroxine injection prolongs the neuroD2-null mouse life span. (A) neuroD2 (arrow) is expressed in pituitary and thyroid/parathyroid tissues as determined by Northern blot analysis. (B) Hematoxylin-eosin staining verified sections covering thyroid and parathyroid glands. (C) Immunostaining by neuroD2 antibody showed that neuroD2 is expressed highly in thyroid follicles (thyrocytes) and partially in epithelial cells (C cells) (arrows) lined around thyroid follicles. (D and E) Detection of the transcription level of thyroglobulin from thyroid gland by radiolabeled RT-PCR with internal control GAPDH. (F and G) X-Gal staining for thyroid tissues and counterstaining with Fast red showed that neuroD2-expressing cells in thyrocytes are not lost in neuroD2-null mice. (H) Serum thyroxine level (UG/DL) detection in five sets of littermates. Thyroxine levels were lower in null mice than in wild-type littermate (wild type, 1.9 ± 0.6; null, 0.8 ± 0.1). (I) Life span of null mice with or without thyroxine treatment (five sets of littermates). All null mice treated with thyroxine lived at least 55 days, compared to a median of 14 days for null littermate mice without treatment. Error bars indicate standard deviations.

the thyroid gland and converted into circulating thyroxine by TSH stimulation. Because neuroD2 is expressed in thyroid gland where thyroglobulin is stored and processed, we determined whether the level of thyroglobulin is altered by RT-PCR. Real-time analysis with SYBER green and radiolabeled RT-PCR revealed decreased thyroglobulin mRNA levels in null thyroid tissue compared to wild type (Fig. 4F) (null is 30% ± 7% of wild type). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control (Fig. 4G).

TSH, also known as thyrotropin, stimulates the thyroid gland to release thyroid hormone; therefore, serum levels of thyroxine (T4) were examined. Serum thyroxine levels were low in neuroD2-null mice compared to wild-type littermates (Fig. 4H). The low serum T4 level in neuroD2-null mice prompted us treat neuroD2-null mice with replacement doses of thyroxine to determine whether any discernible part of the neuroD2-null mouse phenotype was rescued by thyroxine replacement. Daily injection of thyroxine (1 μg/10 g of mouse body weight) (10) into paired littermates began at age P7. The treated mice remained severely neurologically impaired and did not gain appreciable weight despite therapy. In the absence of exogenous thyroxine, the median age of death in neuroD2-null mice was P14 (range, P7 to P21; $n = 41$). All of the mice treated with thyroxine lived for more than 55 days (Fig. 4I). At the 55-day time point, these mice were sacrificed because of neurologic decline. This experiment showed that thyroxine rescued the early-death phenotype in neuroD2-null mice.

DISCUSSION

neuroD2 was originally described as a transcriptional regulator of neuronal differentiation (5, 16). Subsequently, neuroD2 knockout mice showed failure to thrive, small brains, reduced seizure threshold, and aberrant development of hippocampus and cerebellum (20). We recently showed that the basolateral amygdala fails to develop in neuroD2-null mice and has fewer neurons in heterozygous mice. Consistent with this, neuroD2-heterozygous mice show reduced capacity for emotional learning and have a severely impaired innate fear response (13). In these mice, the AMPA and GABA A receptor γ neurotransmitter receptors were reduced in amygdala, and ulip, which is involved in synaptic remodeling, was also diminished. Excitatory neurotransmission is also altered in cortex, which is related to the observation that thalamocortical neurons fail to segregate in neuroD2-null mouse cortex (9a).

The key finding in this study is that the absence of neuroD2 in mice affects function at all levels of the hypothalamic-pituitary-thyroid axis. Unlike the case for amygdala, where neuroD2-positive nuclei fail to form during development in neuroD2-null mice, the populations of cells that express TRH, TSH, and thyroglobulin are present in neuroD2-null and heterozygous mice. TRH mRNA staining was completely absent in the neuroD2-positive neurons of the PVN but was normal in neuroD2-negative neurons in other hypothalamic nuclei. Previous studies have indicated that the TRH neurons in the PVN regulate TSH secretion, while the others do not. In this study, TSH levels were diminished approximately twofold but were not zero, suggesting that TSH is positively regulated by another mechanism(s). In another study, in which TRH was genetically disrupted in mice, TSH immunostaining was decreased in pituitary, yet serum TSH levels were nearly double those in wild-type mice (26). Serum TSH levels could not be measured in the current study because the total amount of serum available from each growth-arrested neuroD2-null mouse was only a fraction of the amount needed for a reliable assay.

In TRH-deficient mice, brains appeared to develop normally and the mice had only a transient decrease in weight around 4 weeks of age. This suggests that the growth retardation affects of congenital hypothyroidism are not modeled well in mice. In fact, growth retardation is variable in human patients with cretinism. The absence of brain abnormalities and growth retardation in TRH-deficient mice raises the possibility that these features in neuroD2-null mice are not secondary to hypothyroidism. Consistent with the absence of growth retardation in TRH-deficient mice, the neuroD2-null mice failed to gain appreciable weight when treated with thyroxine.

We have begun to explore alternative explanations for impaired growth. Growth hormone and IGF2 levels were normal in neuroD2-deficient mice. IGF2 was selected because pilot studies with P19 cells suggested that it is a transcriptional target of neuroD2. IGF1 levels will be evaluated as sufficient serum becomes available. We also conducted glucose tolerance tests. These studies indicated that pancreatic insulin regulation was intact (data not shown).

Hypothyroidism contributes to the severe early-death phenotype of neuroD2-null mice as evidenced by the prolonged survival of mice treated with thyroxine. This was an unexpected finding. Humans with cretinism do not typically die in childhood, nor do TRH- and thyroid hormone receptor-deficient mice. Our interpretation is that hypothyroidism in neuroD2-null mice is one of several factors that contribute to death and that conditional knockout of neuroD2 in the HPT axis alone would be unlikely to yield the same phenotype.

A key question that remains to be answered is whether neuroD2 polymorphisms, mutations, or aberrant function contribute to congenital hypothyroidism in humans. Some patients with congenital secondary hypothyroidism have hypothalamic or pituitary hypoplasia. In certain instances, these are associated with other midline defects, suggesting a defect in midline patterning that is unlikely to be related to neuroD2. In the remaining cases, it would be interesting to learn whether there are mutations in the neuroD2 gene or other genes that cooperate with neuroD2 to regulate the HPT axis. The current study provides a foundation for directed studies with human patients.

ACKNOWLEDGMENTS

We thank Matthew Fero and Wei-Ming Chien for technical advice and critical discussion and Gad Kletter for helpful comments on the manuscript.

This project was supported by NIH grants AR45113 and NS36086 to S.J.T. and by the Burroughs Wellcome Career Award in Biomedical Science and institutional startup funds to J.M.O.

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