Introduction
Thyroid hormones (THs) are essential for normal vertebrate development. In mammals, including humans, precise levels of THs during fetal and neonatal life are critical for appropriate cell proliferation and differentiation (1). Reduced or excessive THs during these developmental stages can result in severe abnormalities (2, 3). Particularly dramatic is the observation of important alterations in the maturation and function of the CNS in mammals with congenital hypothyroidism (2, 3). Some of the important actions of THs during development occur at a time when TH levels are much lower than those in the mother (4, 5) and the hypothalamic-pituitary-thyroid (HPT) axis is not fully functional.

Type 3 deiodinase (D3), a conserved selenoprotein coded by the Dio3 gene, is responsible in part for the low TH levels in the fetus. Dio3 is subject to genomic imprinting and is preferentially expressed from the paternal allele in the mouse fetus (6, 7). D3 catalyzes the conversion of the hormones secreted by the thyroid, the active hormone 3,3',5'-triiodothyronine (T3) and prohormone 3,5,3',5'-tetraiodothyronine (T4), into biologically inactive metabolites by removing an iodine atom from the tyrosyl ring of both compounds to form 3,3'-diiodothyronine (3,3'-T2) and 3,3',5'-triiodothyronine (reverse T3) (8, 9). Thus, D3 is an inactivator of THs and serves as a modulator of intracellular TH levels and TH action.

In rodents, D3 activity is abundant in the pregnant uterus and placenta (10–13) and most fetal tissues (14), including the CNS (15). In contrast, during late neonatal and adult life, D3 activity is more limited, being significant in the skin (16) and central nervous system (15, 17, 18), detectable in certain endocrine organs (18), and very low or absent in other tissues.

Although the physiological significance of D3 is unknown, its biochemical function and tissue expression pattern suggest that it plays a role in protecting tissues, particularly those in the developing fetus, from inappropriate levels of THs. This is of particular importance as fetal serum levels of THs in the rat are only 5% of those present in the mother (19). As the HPT axis matures during late gestation and the neonatal period, serum TH levels rise steadily and reach values comparable to those in the adult in the second week of life (5, 19).

In order to characterize the physiological relevance of D3, we have disrupted the gene coding for D3 in mouse embryonic stem cells and generated a D3 KO mouse (D3KO) with no detectable D3 activity. The D3KO mouse manifests marked abnormalities in thyroid status and physiology, underscoring the critical role of this enzyme in the development and function of the HPT axis.

Results
Neonatal hypothalamic D3 expression. To understand the potential role of D3 in the developing HPT axis, we first determined D3 activity in the mouse hypothalamus during neonatal life. We observed a high level of expression during the first week of life, with a marked diminution immediately thereafter (Figure 1). This pattern is similar to that described in the rat (20) and suggests a role for D3 in limiting hypothalamic exposure to THs during early development.

Generation of D3-deficient mice. We have recently described a successful strategy utilizing homologous recombination in mouse ES cells for disruption of the Dio3 gene (6). The neomycin cassette used for positive selection of recombinant ES cells was excised with appropriate breeding with a Cre-expressing mouse as described in Methods. After this excision, the final structure of the mutated Dio3 locus is illustrated in Figure 2A. The mutant mice carry a triple point mutation affecting 2 codons, one of them coding for...
selenocysteine, an active site residue that is critical for enzyme function (8, 21). That this construct codes for an inactive enzyme was shown by transfection experiments in COS-7 cells (data not shown), indicating that the mutation completely abolished enzyme activity. In addition to the triple mutation, mutant mice carry a residual insertion of a loxP site, 34 bp in length, located in the 3′-untranslated region. The presence of this insertion was used for routine genotyping as illustrated in Figure 2B.

This targeting strategy was designed to fully inactivate D3 while at the same time producing a minimal disruption in the Dio3 locus. This is critically important for 2 reasons. First, this locus is imprinted and belongs to a larger imprinted domain in which long-range mechanisms may control gene expression (22, 23). Second, an additional gene, termed Dio3os, is expressed from the opposite DNA strand and features multiple transcripts that result from alternative splicing (6, 24). The full structure and function of the Dio3os gene have not yet been determined, but partial exonic sequences from a specific Dio3os transcript lie within the Dio3 exon and promoter, S′ to the point mutations and the residual loxP site (24).

Thus, a large deletion in the Dio3 locus might result in unwanted effects in gene expression within this imprinted region or in the disruption of Dio3os gene expression. To confirm that this was not the case, we performed Northern blot analysis to determine Dio3os mRNA expression in fetuses homozygous for the Dio3 mutation. As shown in Figure 2C, there is no noticeable change in the pattern of Dio3os transcripts, suggesting that the small modifications introduced in the Dio3 locus do not disrupt the expression of Dio3os transcripts in these mice. This is consistent with our previous observations that no Dio3os transcripts are detected by Northern blot analysis when using as a probe a genomic fragment comprising the Dio3 3′-untranslated region (24), where the residual loxP site is located. Although it is uncertain whether the triple point mutation introduced in the Dio3 coding region lies within exonic sequence of the Dio3os gene, this mutation would not disrupt any of the potential open reading frames coding for a hypothetical Dio3os protein.

D3 activity is undetectable in D3KO mice. Unless stated otherwise, all the WT and D3KO animals (homozygous for the mutated allele) used in the present work were born to heterozygous mothers of the 129/Sv strain. D3 activity in WT and D3KO mice was determined in various tissues known to express D3, such as the pregnant uterus, placenta, adult midbrain, cerebral cortex and ovary and in E14.5 whole fetuses (Table 1). As expected, no D3 activity was detected in tissues from D3KO mice except in the placenta. Low levels of D3 activity (2.5% of that in the WTs) were measured in the placentas of D3KO fetuses that were conceived by heterozygous mothers. This can be attributable to the presence in the tissue of a residual cell population of maternal origin that expresses D3. Indeed, no placental D3 activity was found in D3KO fetuses that were carried by D3KO mothers (Table 1). These results demonstrate that the introduced mutation completely inactivates the D3.

**General phenotype of the D3KO mouse.** The proportion of D3KO pups obtained from heterozygous matings was lower than the 25% expected from Mendelian laws. Out of 349 newborns produced from heterozygous parents, only 61 (17.5%) were D3KO (P = 0.05). This observation suggests partial lethality of D3KO mice that occurs at or before the time of birth. D3-deficient mice also exhibited impaired reproductive function. Fertility rates were very low in D3KO mice of both sexes. In addition, both male and female D3KO mice were markedly growth retarded (Figure 3). This retardation was already apparent at weaning, when their weight was only 65% of that of the WT mice. This reduction in size persists into adulthood and is still observed in 1-year-old animals (data not shown). Body length is approximately proportional to weight, as shown in the picture included in Figure 3. This general phenotype, with slight variations, is observed in 129/Sv, C57BL/6, and mixed 129/Sv/C57BL/6 genetic backgrounds.

**Weanling and adult D3KO mice manifest central hypothyroidism.** In the late postnatal period, D3KO mice were hypothyroid. Compared with WT mice, the serum T4 level in D3KO weanlings was reduced

<Figure 1>

**Neonatal hypothalamic D3 activity.** Each point represents the mean ± SEM of determinations in 4 animals.

<Figure 2>

**Targeting of the Dio3 locus.** (A) Diagram of the modified Dio3 exon after gene targeting and neomycin cassette excision. The triple point mutation introduced into the Dio3 catalytic site as well as the location of the PCR primers P1 and P2 used for genotyping are shown. (B) Typical results from WT, heterozygous (het), and homozygous mice using the primers P1 and P2. (C) Dio3os mRNA transcripts expressed in WT and D3KO E15.5 fetuses. Thr, threonine; Secys, selenocysteine.
by more than 95%, and the serum T3 concentration was reduced by 50% of normal. However, the serum thyroid-stimulating hormone (TSH) level was unaffected (Figure 4A). In adult D3KO mice, serum T4 and T3 levels were also low (27% and 80% of those in WT animals, respectively) while the serum TSH level was elevated 50% (Figure 4B). A very similar pattern of thyroid parameters was observed in older adults (Figure 4C), indicating that the central hypothyroidism persists through adult life. The increase in the TSH level in adults was much lower than what was anticipated, given the low circulating levels of THs. As a comparison, a 90-fold increase in TSH concentrations has been observed in mice in which comparably low TH levels were induced by feeding a low-iodine diet containing propylthiouracil (25). This failure of the serum TSH level to be elevated appropriately in the face of low circulating T4 and T3 levels points to a central etiology of the hypothyroidism. D3KO mice were thyrotoxic early in life with markedly diminished T3 clearance, and this likely plays an important role in the very low serum T4 levels observed in adult D3KO mice in which comparably low TH levels were induced by feeding a 90-fold increase in TSH concentrations has been observed in mice in which comparably low TH levels were induced by feeding a low-iodine diet containing propylthiouracil (25). This failure of the serum TSH level to be elevated appropriately in the face of low circulating T4 and T3 levels points to a central etiology of the hypothyroidism. D3KO mice were thyrotoxic early in life with markedly diminished T3 clearance, and this likely plays an important role in the very low serum T4 levels observed in adult D3KO mice in which comparably low TH levels were induced by feeding a 90-fold increase in TSH concentrations has been observed in mice in which comparably low TH levels were induced by feeding a low-iodine diet containing propylthiouracil (25). This failure of the serum TSH level to be elevated appropriately in the face of low circulating T4 and T3 levels points to a central etiology of the hypothyroidism.

Table 1

D3 activities in different tissues of WT and D3KO mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WT</th>
<th>D3KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant uterus</td>
<td>12402 ± 552</td>
<td>Undetectable</td>
</tr>
<tr>
<td>E14.5 fetus</td>
<td>988 ± 92</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Adult cortex</td>
<td>1024 ± 42</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Rest of adult cerebrum</td>
<td>1066 ± 102</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Adult ovary</td>
<td>73 ± 3</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Placenta (heterozygous mother)</td>
<td>3070 ± 267</td>
<td>77 ± 6</td>
</tr>
<tr>
<td>Placenta (D3KO mother)</td>
<td></td>
<td>Undetectable</td>
</tr>
</tbody>
</table>

D3 activities (fpmol/mg protein) represent the mean ± SEM. Uterus and placenta samples correspond to E17.5 gestational age. Adult tissues were obtained from 3-month-old mice. From 4 to 6 samples were used for each determination. Undetectable activities in the assay conditions were lower than 2 fpmol/mg protein.

To gain further insight into the cause of the high T3 levels observed during the perinatal period in D3KO mice, we evaluated the rate of T3 clearance from the serum. WT and D3KO 2-day-old pups were injected with a tracer amount of [125I]-T3, and the [125I]-T3 present in the blood was measured at different times. Two hours after injection, the level of [125I]-T3 in the serum of D3KO pups was double that in the WT mice (Figure 7A). Seven hours after injection, [125I]-T3 had decreased significantly in WT pups but very little in the D3KO pups. From the slopes of the curves, we estimated that D3KO newborns exhibited a significant reduction in the serum T3 clearance rate. Serum [125I]-T3 in WT mice decreased approximately 5 times faster than in D3KO animals. Small amounts (<5% of the total) of other radioactive metabolites were noted in the serum of injected animals. In addition, values for serum T3 uptake were comparable in WT and D3KO mice at P2 (Figure 7B), suggesting no difference in serum binding of T3 between the 2 strains. Thus D3KO neonates demonstrate a diminished T3 clearance, and this likely plays an important role in the high serum level of T3 observed at this age.

Brain thyrotoxicosis in D3KO neonates. To determine the effects on the brain of the elevated serum T3 level during the neonatal perinatal period in D3KO mice, we evaluated the rate of T3 clearance from the serum. WT and D3KO 2-day-old pups were injected with a tracer amount of [125I]-T3, and the [125I]-T3 present in the blood was measured at different times. Two hours after injection, the level of [125I]-T3 in the serum of D3KO pups was double that in the WT mice (Figure 7A). Seven hours after injection, [125I]-T3 had decreased significantly in WT pups but very little in the D3KO pups. From the slopes of the curves, we estimated that D3KO newborns exhibited a significant reduction in the serum T3 clearance rate. Serum [125I]-T3 in WT mice decreased approximately 5 times faster than in D3KO animals. Small amounts (<5% of the total) of other radioactive metabolites were noted in the serum of injected animals. In addition, values for serum T3 uptake were comparable in WT and D3KO mice at P2 (Figure 7B), suggesting no difference in serum binding of T3 between the 2 strains. Thus D3KO neonates demonstrate a diminished T3 clearance, and this likely plays an important role in the high serum level of T3 observed at this age.

Figure 3

Postweaning growth curves of WT and D3KO male mice. Each point represents the mean ± SEM of measurements recorded in 7 to 58 animals at each age. Mean and median group size per data point were 18 and 12, respectively. Only data from animals born in litters of 3 to 7 pups are included. Data from extremely growth retarded D3KO mice, which typically do not survive through weaning, are not included. A picture of representative WT and D3KO weanlings is shown. Body length appear to be proportional to body weight.
od, we analyzed the mRNA expression of RC3 and hairless, 2 genes that are upregulated by THs in the CNS during the neonatal period (28, 29). In 1- to 3-day-old total brains, hairless expression was significantly elevated in D3KO animals (Figure 8A). The hairless mRNA level in the hypothalamus of D3KO mice was also markedly elevated at P5 (Figure 8A). These observations indicate that the elevated serum T3 level is accompanied by enhanced T3 action in the neonatal brain. Indeed, the T3 concentration in the brains of D3KO neonates at P2 was more than double that in WT mice (Figure 8B). At P3, RC3 mRNA expression was also stimulated in the brain of D3KO mice as compared with that of WT mice (Figure 8C), but its expression was lower than normal by P15 (Figure 8C), following the onset of central hypothyroidism. These results indicate that the brain evolves from a thyrotoxic state in the newborn to a hypothyroid state in late neonatal life, undergoing a transition in thyroid status analogous to that observed in serum.

An important factor regulating TH action in the brain is type 2 deiodinase (D2), an enzyme that converts the prohormone T4 into the active hormone T3 (8, 9). D2 protects the brain from low levels of THs, and its activity is markedly increased when T4 levels are low (8, 30). In the present study, the profile of D2 activity in the brain of D3KO mice was consistent with the observed low serum T4 level. Thus, D2 activity was significantly elevated in the cerebral cortex of D3KO mice on P5 (Figure 8D) and was dramatically elevated (10- to 15-fold higher than in WT mice) from P10 to P21. A significant increase in D2 activity was also found in the adult cortex at P90.

Thyrotropin-releasing hormone expression and hypothalamic T3 content. To investigate the nature of the central hypothyroidism found in weanlings and adult D3KO mice, we measured hypothalamic T3 content and thyrotropin-releasing hormone (TRH) mRNA expression. At 15 days of age, no difference was observed in the T3 content of the hypothalamicus between WT and D3KO mice (Figure 9A). However, a moderate but significant decrease in the T3 content was observed in the hypothalamus of adult D3KO mice. No difference in TRH mRNA was observed at P15 (Figure 9B), but TRH expression was elevated in adult D3KO mice, consistent with the diminished hypothalamic T3 content observed at this age.

Discussion

We have found that mice lacking an active D3 exhibit a number of abnormalities, including partial perinatal mortality, growth retardation, and impaired fertility. D3-deficient neonatal mice also manifest decreased T3 clearance and striking alterations in serum and tissue TH levels during perinatal life and adulthood. Thus,
our findings in this new mouse model indicate that D3 is critical for both normal development and function of the thyroid axis.

We have shown that the D3KO mouse has no detectable D3 enzymatic activity and that the pattern of transcripts for the Dio3os gene that is transcribed from the opposite strand at the Dio3 locus does not appear to be disrupted. Thus, the abnormalities observed in the D3KO mice likely are due solely to the absence of an active D3 enzyme.

Our observations indicate that the lack of D3 results in a markedly elevated level of T3 in the serum of fetuses and early neonates. As it is during this developmental stage that D3 expression is at its highest in the hypothalamus and in most tissues, our results demonstrate that D3 plays a critical role in maintaining low levels of THs during fetal and early neonatal life. In the absence of D3, the clearance of T3 is diminished, and this likely contributes to the perinatal thyrotoxicosis observed in D3KO mice. This is considered to be due to central abnormalities of the HPT axis. Indeed, the TH parameters in the D3KO mouse are remarkably similar to those observed in the TRH and TRH-receptor KO mouse models (32, 33), where serum T4 and T3 are decreased and serum TSH is modestly elevated or unchanged, respectively.

In the adult D3KO mouse, the inability of increased TSH levels to normalize TH concentrations may reflect a defect in the thyroid gland. Although this possibility cannot be discarded, preliminary observations indicate that the size and appearance of the thyroid gland is normal. A more plausible explanation for the lack of TSH effect is that TSH bioactivity is diminished. TRH signaling is critical for the proper maturation and glycosylation of TSH (34, 35), and these posttranslational modifications have been shown to be necessary for full TSH bioactivity in human cases of central hypothyroidism of hypothalamic origin (36). This has also been shown in the TRH KO mouse (37).

In considering the etiology of the central hypothyroidism in the adult D3KO mouse, the rodent model referred to as the neonatal T4 (neo-T4) syndrome may be of relevance (38, 39). In this model, the injection of pharmacological doses of T4 (or T3) to rats for 3 to 5 days immediately after birth results in the development of central hypothyroidism in adults. These animals also show an

Figure 6
Ontogeny of serum T3, T4, and TSH. (A) Perinatal serum T3 levels. (B) Perinatal serum T4 levels. T4 levels were undetectable (<0.1 µg/dl) in E19.5 fetuses and P1 neonates of both genotypes as indicated by the dotted line. Each point represents the mean ± SEM of determinations in 6 (A) and 7 (B) animals in each group. *P < 0.0001, WT versus D3KO. (C) Ontogeny of T3, T4, and TSH serum levels expressed as a percentage of the values in WT animals of corresponding age. Serum TSH was undetectable in D3KO mice between 5 and 15 days of age (see text for absolute TSH values).
impaired pituitary response to TRH (40, 41). Thus, the neo-T4 rat model demonstrates that exposure to inappropriately high levels of THs before the HPT axis is functionally mature results in pituitary and/or hypothalamic abnormalities that affect its regulatory mechanisms and set point.

Similar observations have been made in humans. Several articles have described infants who experienced thyrotoxicosis in utero as a result of poorly controlled maternal hyperthyroidism and who subsequently developed transient neonatal central hypothyroidism (42, 43). In view of these observations, we suggest that the main cause of central hypothyroidism in D3KO mice is their overexposure to T3 during a critical period of thyroid axis development. The molecular parameters mediating this occurrence have not been identified in any of these models.

Notably, the central hypothyroidism observed in D3KO mice is more severe than that in neo-T4 rats whereas in the human cases mentioned above, the central hypothyroidism appears to be transient. Although common mechanisms may cause the abnormalities of the HPT axis in the 3 models, the increased severity of the D3KO phenotype may be due to a higher degree and/or longer duration of tissue T3 overexposure. In this regard, it is worth noting that both the neo-T4 rats and human infants born to hyperthyroid mothers have functional D3 that may be upregulated in the face of hyperthyroidism (44) and thus partially protect the brain from exposure to high TH levels. D3KO mice have no such protective mechanism to ameliorate overexposure to T3. Hence, a more severe phenotype is not unexpected.

D3KO mice possess intact TRH and TRH-receptor genes and, indeed, TRH expression is increased in the adult D3KO hypothalamus, presumably in response to the demonstrated decrease in T3 content. Thus, D3 deficiency does not lead to excessive T3 effects (e.g., suppression of TRH) in the hypothalamus of the adult D3KO animal. This observation implies that the mechanism or mechanisms responsible for TSH dysregulation in the D3KO animal differ fundamentally from that of the TRH KO animal and involve other molecular changes that alter the set point of TSH secretion in response to TH feedback. Of interest in this regard is that THs are known to regulate TRH-receptor mRNA levels in the pituitary gland, and conceivably this could be part of a develop-

Figure 8
Brain expression of T3-regulated genes. (A) Expression of hairless in the newborn brain and neonatal hypothalamus. Representative Northern blots are shown, and quantification of expression was performed in the number of animals indicated in parentheses. Each bar represents the mean ± SEM. Ribosomal staining or cyclophilin expression was used as a control to correct for the amount of RNA loaded per lane. (B) Brain T3 content of P2 newborns. (C) Northern blot analysis of RC3 expression in the brain at P3 and P15. The most abundant RC3 transcript is shown. (D) Neonatal and adult brain D2 activity. Each point represents the mean ± SEM of determinations in 6 animals. *P < 0.0001, WT versus D3KO.

Figure 9
Hypothalamic T3 content and TRH mRNA expression. (A) Hypothalamic T3 content as determined by RIA in hypothalami of 15-day-old and adult (3-month-old) mice. (B) Quantitation of Northern blot analysis of hypothalamic TRH mRNA levels in 15-day-old and adult mice. Each bar represents the mean ± SEM of determinations in 5 animals. *P < 0.001, WT versus D3KO.
mental program to determine the set point of the thyroid axis control mechanisms (45). The thyroid status of the D3KO hypothalamus at P15 does not reflect the circulating low TH levels; the T3 content of the hypothalamus is not different from that observed in WT mice despite significant decreases in serum T3 and T4 levels. This is possibly due to the marked increase in D2 activity or the fact that the brain is still evolving from a thyrotropic state and has yet to adjust to lower serum TH levels. In the adult, however, the thyroid status of the hypothalamus in the D3KO mouse reflects the hypothroidism level and results in a modest increase in TRH mRNA expression that qualitatively mimics the situation in WT animals (46). This finding of a relatively small increase in TRH expression might be due to the fact that only a fraction of hypothalamic neurons display T3-sensitive TRH expression (45).

Other phenotypic abnormalities observed in the D3KO mouse include impaired growth, low fertility, and partial perinatal lethality. It is well established that THs exert profound effects on growth hormone expression (47). Thus, alterations in the growth hormone axis may play a role in the growth retardation observed in D3KO mice. Impaired viability in D3KO mice may be due to perinatal thyrototoxicosis, as a similar observation has been made in rats and humans (48, 49). Concerning the reproductive function of the D3KO mouse, severe hypothyroidism may affect fertility in both sexes, as demonstrated by hyt/hyt mice (50, 51). Of note, fertility in the TRH KO mouse, which manifests a milder degree of hypothyroidism that is similar to that in the D3KO mouse, is normal (32). Thus, factors in addition to alterations in adult thyroid status likely play a role in the impaired fertility of the D3KO mouse. For instance, in utero thyrotoxicosis may also contribute to the perceived lower fertility of D3KO female mice, as the absence of a functional maternal D3 in tissues involved in implantation and placentation may be detrimental to early embryonic viability.

In summary, our results demonstrate that D3 is critical for the normal development and function of the thyroid axis and plays a role in maintaining appropriate TH levels in the fetus and neonate. D3 may be expressed to lower T3 content in this region such that the thyroid axis set point can develop normally. Beyond the neonatal period, the rapid decrease in D3 activity allows the hypothalamus to more accurately track serum TH levels and thus adjust TRH expression appropriately.

Although a D3 deficiency has not yet been reported in humans, the observations in the D3KO mouse predict the occurrence of central hypothyroidism as part of the phenotype in such cases. This new model of central hypothyroidism will be valuable for analyzing the events regulating the maturation and function of the thyroid axis, as well as the role of D3 and THs in the physiology of growth, development, and reproduction.

Methods

Generation of D3KO mice. We recently described (6) the strategy used to target the Dio3 gene using standard homologous recombination techniques. We utilized the R1 ES cell line (52), which originated from the 129/Sv mouse strain. Targeted clones were identified by Southern blot analysis, injected into C57BL/6 blastocysts, and reimplanted in CD1 foster mothers. Chimeric males that showed germ-line transmission were mated to C57BL/6 female mice to test for germ-line transmission of the mutation. Chimeric males were then mated with 129/Sv females to establish the mutant line in a 129/Sv background. The neomycin cassette was excised by mating heterozygous females with a 129/Sv male carrying in chromosome X a transgene expressing the Cre DNA recombinase. The removal of the neomycin cassette was confirmed by Southern blot analysis in the first generation females. The Cre DNA recombinase transgene was removed from the genetic background of the colony by appropriate matings with WT 129/Sv animals and sex selection. Animals were kept under a 12-hour light cycle and provided food and water ad libitum. Animal procedures were approved by the Dartmouth College Institutional Animal Care and Use Committee.

Mice genotyping and serum and tissue sampling. After the removal of the neomycin cassette, genotyping of mice carrying the inactivating mutation was performed by PCR amplification of the residual loxP site (Figure 1). The primers used were as follows: 5′-GGAGTCCCTGTGCTTTTG-3′ (sense); 5′-CGACCTCTCTGCAATTAG-3′ (antisense). The PCR protocol consisted of 32 cycles that included 20 seconds at 94°C, 20 seconds at 60°C, and 45 seconds at 72°C, with a final extension of 3 minutes. Mouse DNA was isolated by standard procedures after proteinase K digestion of tail snips.

Animals were killed by asphyxiation with CO2 (adults and weanlings) or by decapitation (neonates). In the adults and older neonates, blood was taken from the superior vena cava while trunk blood was collected from younger neonates and fetuses. Serum was obtained by centrifugation and stored at –20°C. For Northern blot analysis and enzymatic activity, tissues were disected, immediately frozen on dry ice, and stored at –70°C. Whole hypothalami were dissected, considering the midbrain and thalamus as the posterior and dorsal limits, respectively, and the optic chiasm and its ends as the anterior and lateral limits, respectively.

For the serum determination of T3 clearance, 2-day-old neonates were injected intraperitoneally with a trace amount of [125I]-T3 (New England Nuclear) (150,000 cpm, approximately 100 fmol in a volume of 50 μl). Trunk blood was collected at 2 and 7.5 hours after injection and centrifuged to obtain the serum that was then subjected to paper chromatography to separate T3 from other radioactive metabolites as described (18). The amount of radioactivity attributable to T3 was determined with a gamma counter. The clearance rate of T3 in serum was estimated from the slopes of the lines obtained by plotting the amount of residual [125I]-T3 against the time after injection. The amount of [125I]-T3 injected was not corrected by body weight, as at 2 days of age the weight of D3KO mice is within 10% of that in WT animals.

D1, D2, and D3 activities. D1, D2, and D3 enzymatic activities were determined as previously described (18, 44). In brief, tissues were homogenized in a 10 mM tris-HCl, 0.25 sucrose pH 7.5 buffer. A suitable volume of tissue homogenate was used in the enzymatic reaction to ensure that deiodination did not exceed 20% and was proportional to the amount of protein content. Tissue homogenates were incubated at 37°C for an hour with the appropriate [125I]-labelled iodothyronine (New England Nuclear). For the D1 assay, 400 nM of reverse T3 in the presence of 2 nM of the cofactor DTT were used. For the D2 assay, we used 1 nM T4 and 20 mM DTT. For the D3 assay, 2 nM T3 and 20 mM DTT were used. Deiodination was determined based on the percentage of labeled iodine released (D1 and D2 assays) or the amount of [125I]-3,3′-diiodothyronine produced (D3 assay). The latter was determined after separation of reaction products by paper chromatography, as described (53). A factor of 2 was included in the calculations of D1 and D2 activities to correct for the chemical equivalence of the outer ring iodine residues and the fact that only 1 of them is labeled in a given molecule.

RNA preparation and Northern blot analysis. Total RNA and poly (A+) RNA were isolated from mouse liver by guanidine hydrochloride and oligo-dT cellulose methods, respectively, following standard procedures (54). Total RNA was isolated from brain tissues using the Ribopure kit from Ambion Inc. Total and poly (A+) RNA samples were electrophoresed in a denaturing 1% agarose gel containing formaldehyde and blotted onto a Nyttran membrane (Schleicher & Schuell). Blots were hybridized at 42°C in buf-
fer containing 50% formamide, washed with 0.1X SSC/0.1% SDS at 65°C, and autoradiographed for 1 to 7 days. Probes were labeled with radioactive 32P-dCTP (ICN Biochemicals Inc.) using the Oligolabelling Kit (Pharmacia Corp.) and were purified through G-50 columns (Pharmacorp.). Quantification of mRNA bands was performed by computer-assisted densitometry (Molecular Dynamics). The mouse cDNA probes used were as follows: hairless, a 3-kb BamHI fragment that includes most of the coding region; RC3, the complete 1.3-kb cDNA; TRH, a 0.8-kb PCR fragment that includes the coding region; S14, the complete 1.3-kb cDNA; D1, the complete 1.7-kb cDNA; Diao3, a mix of 3 partial cDNAs with GenBank accession numbers AY283182, AY283181, and AY077459.

**Hormone determinations.** Serum total T4 concentration was determined using the Total T4 Coat-A-Count RIA kit (Diagnostic Systems Laboratories Inc.) according to the manufacturer's instructions. The sensitivity of the assay as determined experimentally ranged from 0.1 to 0.2 µg/dl. The serum T3 level was determined using a sensitive RIA method established in our laboratory (55) with the modification that the T3 antibody used was obtained from a commercial source (Fitzgerald Industries International Inc.). An index of the circulating levels of TH carrier proteins was obtained by measuring the residual capacity of the serum to bind [125I]-T3, using the Coat-A-Count T3 uptake kit (Diagnostic Systems Laboratories Inc.) according to the manufacturer's instructions. For brain and hypothalamic T3 determinations, the tissue was weighed and homogenized in 2 ml of methanol containing 1 mM propylthiouracil, centrifuged, and the pellet reextracted twice more. Methanol from the supernatants was collected and evaporated, the residue resuspended in a buffer containing 0.2 M glycine, 0.13 M sodium acetate, and 0.02% bovine serum albumin, and analyzed by RIA. Recoveries were not considered for the calculations as they were determined to be higher than 95% by using a radioactive tracer. T3 was calculated as the average of determinations at 2 different dilutions that typically did not differ more than 15%. The cross reactivity of T4 with the T3 anti-body was less than 0.38%. Adult and weanling serum TSH levels were determined using a highly sensitive double-antibody method developed by A.F. Parlow (56). Cross reactivity with follicle-stimulating hormone or luteinizing hormone was less than 1%. Neonatal serum TSH was measured in 50 µl of serum using a sensitive, heterologous, disequilibrium, double-antibody precipitation radioimmunoassay developed by Pohlenz et al. (57).

**Statistics.** Statistical significance between groups was determined by the 2-tailed Student's t test. To assess the proportions of genotypes in the offspring from heterozygous matings, statistical significance was determined by the χ2 test.

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