0888-8809/01/$03.00/0
Printed in U.S.A.

Molecular Endocrinology 15(12):2137–2148
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Targeted Disruption of the Type 2 Selenodeiodinase Gene (*DIO2*) Results in a Phenotype of Pituitary Resistance to T_4

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The type 2 deiodinase (D2), a selenoenzyme that catalyzes the conversion of T_4 to T_3 via 5′-deiodination, is expressed in the pituitary, brain, brown adipose tissue (BAT), and the reproductive tract. To examine the physiological role of this enzyme, a mouse strain lacking D2 activity was developed using homologous recombination. The targeting vector contained the *Neo* gene in place of a 2.6-kb segment of the *Dio2* gene. This segment comprises 72% of the coding region and includes the TGA codon that codes for the selenocysteine located at the active site of the enzyme. Mice homologous for the targeted deletion [D2 knockout (D2KO)] had no gross phenotypic abnormalities, and development and reproductive function appeared normal, except for mild growth retardation (9%) in males. No D2 activity was observed in any tissue in D2KO mice under basal conditions, or under those that normally induce this enzyme such as cold-exposure (BAT) or hypothyroidism (brain, BAT, and pituitary gland). Furthermore, no D2 activity was present in cultured astrocytes, nor could it be induced by treatment of the cells with forskolin. Although D2 mRNA transcripts were detected in BAT RNA obtained from cold-exposed wild-type (WT) mice, none was detected in BAT RNA from comparably-treated D2KO mice. Levels of D1 in the liver, thyroid, and pituitary were the same in WT and D2KO animals, whereas D3 activity in D2KO cerebrum was twice that in WT cerebrum. Serum T_3 levels were comparable in adult WT and D2KO mice. However, serum T_4 and TSH levels were both elevated significantly (40% and 100%, respectively) in the D2KO mice, suggesting that the pituitary gland of the D2KO mouse is resistant to the feedback effect of plasma T_4. This view was substantiated by the finding that serum TSH levels in hypothyroid WT mice were suppressed by administration of either T_4 or T_3, but only T_3 was effective in the D2KO mouse. The data also suggest that the clearance of T_4 from plasma was reduced in the D2KO mouse. In summary, targeted inactivation of the selenodeiodinase *Dio2* gene results in the complete loss of D2 activity in all tissues examined. The increased serum levels of T_4 and TSH observed in D2KO animals demonstrate that the D2 is of critical importance in the feedback regulation of TSH secretion. (*Molecular Endocrinology* 15: 2137–2148, 2001)

DEIODINATION IS THE major mechanism of thyroid hormone metabolism in vertebrates (1, 2). Three types of deiodinase activity have been identified and the cDNAs for these enzymes, designated types 1, 2, and 3 (D1, D2, and D3), have been cloned from several species (3–6). All three enzymes are selenoproteins and contain the rare amino acid selenocysteine at their active sites. D1 and D2 are primarily activating enzymes in that they catalyze the 5′-deiodination (5′D) of T_4 to the metabolically more active product T_3. D3 is an inactivating enzyme; it catalyzes the 5-deiodination (5D) of both T_4 and T_3 to their relatively inactive derivatives, rT_3 and 3,3′-diiodothyronine, respectively. D1 also catalyzes 5D with the preferred substrates for this activity being the 4′-sulfated conjugates of T_4 and T_3 (7).

D1 and D2 can be distinguished by their kinetic properties, substrate preferences, patterns of inhibition by compounds such as 6n-propyl-2-thiouracil (PTU) and aurothioglucose, and their response to changes in thyroid status (1, 2). They also exhibit different profiles of expression in tissues; the highest levels of D1 activity are found in liver, kidney, and thyroid gland while the highest levels of D2 activity are found in pituitary, brown adipose tissue (BAT), and the central nervous system (CNS) (1, 2).

There is indirect evidence to indicate that the deiodinases play a role in the tissue- and cell-specific regulation of intracellular levels of the active thyroid hormone, T_3. For example, the finding that the ratio of T_3 to T_4 varies widely among tissues, but is invariably higher than that in plasma, is likely dependent, at least

Abbreviations: BAT, Brown adipose tissue; CNS, central nervous system; D1, D2, D3, types 1, 2, and 3 deiodinase, respectively; 5D, 5-deiodination; 5′D, 5′ deiodination; D2KO, D2 knockout; DTT, dithiothreitol; ES, embryonic stem; MMI, methimazole; PTU, 6n-propyl-2-thiouracil; TPA, tetradecanoyl-phorbol-13-acetate; WT, wild-type.

in part, on the relative levels of activity of the three enzymes in each tissue (8). There is also evidence that the deiodinases are involved in the adaptation of the organism to environmental and internal challenges. Changes in the activities of one or more of the deiodinases have been noted in starvation, illness, exposure to cold, and changes in thyroid status (1, 2, 6, 9).

However, the precise physiological roles of the individual deiodinases, in particular D1 and D2, which both catalyze 5′D, have not been clearly defined. It has been estimated that D1 activity generates T_3 from T_4 primarily for export to the plasma (10, 11), while D2 activity is thought to generate T_3 mainly for local use in tissues. In fact, the presence of D2 in tissues such as pituitary, brain, and BAT has led to the view that it plays a key role in regulating thyroid hormone-dependent processes in these organs, in particular the pituitary feedback mechanism, developmental processes in brain, and thermogenesis in BAT (12–14). But proof of this concept of separate functions for the two enzymes is complicated by the fact that pituitary and brain express D1 as well as D2, and evidence has been obtained that some of the T_3 generated by D2 is also exported to the plasma (15).

One approach to determining the function of an individual deiodinase would be to inhibit specifically its activity. Unfortunately, there is no known pharmacological agent that will specifically and completely inhibit the activity of any of the deiodinases individually. Iopanoic acid is an excellent inhibitor but it inhibits the activity of all three of them. PTU can be employed at levels that greatly reduce the activity of D1 while having little effect on that of D2, but the inhibition is not complete. Furthermore, this compound also inhibits thyroid hormone biosynthesis, and thus it is unsuitable for studies *in vivo* in animals where intact thyroid function is required.

A second approach is to create an animal model that lacks the active form of a deiodinase enzyme by, for example, targeted disruption of a specific deiodinase gene. We have used this technique successfully to create a mouse that is completely deficient in D2 activity. The methods used in its production, the data confirming that the gene disruption has led to a complete loss of D2 activity in tissues, and details concerning the resulting phenotype are presented herein.

RESULTS

Initial Characterization of D2KO Mice

Matings of heterozygote male and female mice yielded heterozygotes, WTs, and D2KOs at approximately the expected frequency (50%, 25% and 25%), and no gross physiological or behavioral abnormalities were observed in the offspring. Male and female fertility in the D2KO mice appeared to be normal, and litter size was unaffected; mean litter sizes in 58 WT and 48 D2KO pregnancies were 7.94 ± 0.45 and 7.41 ± 0.42, respectively; the difference was not significant. However, some differences were noted in body weight. Before weaning at 3 wk, mean body wt in the D2KOs and WTs was comparable, but from 4 to 7 wk the mean body wt was lower in male D2KOs than in male WTs, and the difference, although small (9%), was highly significant (Table 1). However, by 8 wk no significant difference was observed. The weights of female D2KO and WT mice were comparable at all ages studied (data not shown). D2KO mice were also able to survive exposure to cold (4 C) for 8 d.

D2 Activities in Tissues

To ascertain that the mice carrying two copies of the recombinant allele did not exhibit any D2 activity, D2KO and WT mice were subjected to treatments known to enhance D2 activity. First, mice were exposed to 4 C for 4 h, a condition that results in a marked increase in D2 activity in BAT (16). After this treatment, BAT from the WT mice exhibited substantial D2 activity, but none was observed in BAT from the D2KO mice (Fig. 1). D2 activity in BAT from heterozygotes was approximately 50% of that in WT BAT. To be certain that the lack of D2 activity in D2KO BAT was not due to a constitutive property of one or both of the background strains of mice, D2 activity was also determined in WT 129SV and C57Bl/6 mice after com-

Table 1. Body Weight in Male WT and D2KO Mice from 4–8 Wk

	Age				
	4 wk	5 wk	6 wk	7 wk	8 wk
Series 1					
WT	17.0 ± 0.74[a]	20.7 ± 0.46			25.7 ± 0.59
D2KO	13.5 ± 0.45	18.7 ± 0.47			24.5 ± 0.39
	$P < 0.001$	$P < 0.001$			NS
Series 2					
WT	17.9 ± 0.51		21.6 ± 0.36	23.0 ± 0.48	24.0 ± 0.55
D2KO	16.6 ± 0.48		19.8 ± 0.48	21.2 ± 0.45	22.62 ± 0.49
	NS		$P < 0.001$	$P < 0.01$	NS

[a] Eighteen to 25 mice/group. NS, Not significant, $P > 0.05$.

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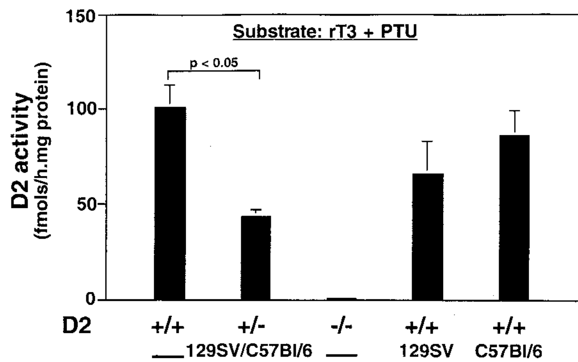

Fig. 1. D2 Activity in BAT from WT and D2KO Mice Exposed to 4 C for 4 h

The substrate was 1 nM rT_3 incubated in the presence of 1 mM PTU. *Bars* indicate the mean ± SE of values obtained in six mice.

parable cold exposure. The levels of D2 activity in BAT from both strains approached that observed in the mixed strain WT BAT (Fig. 1).

Second, D2KO and WT animals were made hypothyroid by administration of drinking water containing methimazole (MMI)/ClO_4. After 5 wk, tissues from these mice were assayed for D2 activity. In WT mice, hypothyroidism resulted in a 3-fold increase in BAT D2 activity as determined using rT_3 in the presence of PTU as substrate (euthyroid BAT: 8.6 ± 2.05; hypothyroid BAT: 33.0 ± 11.5 fmol/h·mg protein, $P < 0.05$). In contrast, no D2 activity was detected in BAT from either euthyroid or hypothyroid D2KO mice. In cerebrum and pituitary, D2 activity was determined using both rT_3 and T_4 as substrates. In pituitary (Fig. 2A) and cerebrum (Fig. 2B) from WT mice, hypothyroidism resulted in a marked increase in D2 activity, and the results obtained with the two substrates were comparable. However, no D2 activity was detected using either substrate in pituitary or cerebrum from either euthyroid or hypothyroid D2KO mice.

Northern analysis of BAT mRNA obtained from cold-exposed D2KO and WT was carried out using the coding region of a mouse D2 cDNA as probe (Fig. 3). A strong signal was obtained at approximately 7 kb, the known size of mouse D2 transcripts (17), in the lane containing the WT mRNA. No comparable signal was observed in the lane containing the D2KO mRNA. When the blot was stripped and reprobed for cyclophilin transcripts as a positive control, a signal at approximately 0.8 kb was obtained in both lanes and the signal was stronger in the lane containing the D2KO RNA.

D2 Activity in Cultured Astrocytes

D2 activity is present at low levels in preparations of cultured astrocytes from neonatal rodents, and the activity is markedly increased in the presence of cAMP (18) and compounds such as forskolin, which activate adenylyl cyclase (19). The phorbol ester, tetra-

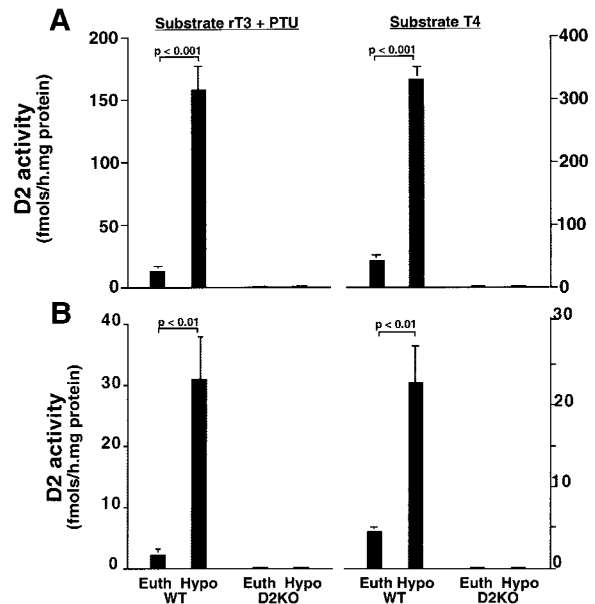

Fig. 2. D2 Activity In Pituitary (A) and Cerebrum (B) from Euthyroid and Hypothyroid WT and D2KO Mice

Hypothyroidism was achieved by placing the mice on drinking water containing 0.1% MMI and 1% $KClO_4$ for 5 wk before study. Results obtained using 1 nM rT_3 as substrate, incubated in the presence of 1 mM PTU, are shown on the *left*, and those using 1 nM T_4 as substrate are shown on the *right*. *Bars* indicate the mean ± SE of values obtained in six (hypothyroid) and eight (euthyroid) mice.

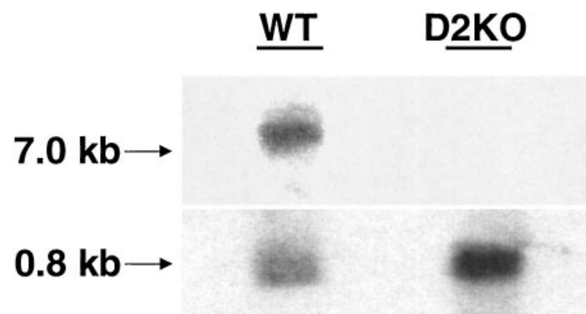

Fig. 3. Northern Analysis of D2 mRNA Transcripts in BAT Poly(A)$^+$ RNA Prepared from WT and D2KO Mice After 16 h Exposure to 4 C

WT and D2KO lanes were loaded with 4.8 and 6.0 μg mRNA, respectively. The blot was probed first with the coding region of the mouse D2 cDNA (*upper panel*) and then with the cDNA for the control gene, cyclophilin (*lower panel*).

decanoyl-phorbol-13-acetate (TPA), also stimulates D2 activity, but to a lesser extent than does forskolin, and only in the presence of glucocorticoid (20). In the present study, astrocytes from 2-d-old WT and D2KO mouse brains were cultured and D2 activity was determined after exposure of the cells to forskolin or TPA. In astrocytes from WT mice, D2 activity was increased 12-fold and 26-fold after exposure to forskolin for 4 and 6 h, respectively; the 2-fold increase

that occurred after exposure to TPA for 8 h was not statistically significant (Fig. 4). In contrast, no D2 activity was detected in the astrocytes from the D2KO mice in the presence or absence of either compound.

D1 and D3 Activity in Tissues of D2KO Mice

D1 activity was measured in liver, thyroid, and pituitary to determine whether the expression of this enzyme was altered in the D2KO mice to compensate for the lack of D2 activity (Fig. 5). In all three tissues, there was no significant difference between the levels of D1 activity in euthyroid D2KO and WT mice. D1 activity was markedly decreased in liver and substantially increased in thyroid and pituitary from both hypothyroid D2KO and WT mice. However, while the increase in D1 activity in thyroid was comparable in the hypothyroid D2KO and WT mice, in pituitary the increase was much less marked in the D2KO than in the WT mice.

5D activity was determined in the cerebral cortex of euthyroid and hypothyroid D2KO and WT mice. The assays were carried out in the presence and absence of PTU to assess the contributions of the D3 and D1 enzymes. The majority of the 5D activity was PTU insensitive (WT 62%, D2KO 82%) and thus represented D3 activity (Fig. 6). The PTU-sensitive activity was assumed to be the result of the inner-ring deiodinating capacity of the D1 enzyme, which is sensitive to PTU (21). The level of D3 activity was more than 3-fold higher in euthyroid D2KO cortex than in cortex of WT mice. Hypothyroidism did not influence the level of D3 activity in the WT cortex, but that in the D2KO cortex, while still significantly higher than that in WT cortex, was reduced. Mean values for D1 activity were also higher in the D2KO than in the WT cortex, both in the euthyroid and the hypothyroid mice, but the difference reached statistical significance only under the latter condition (Fig. 6).

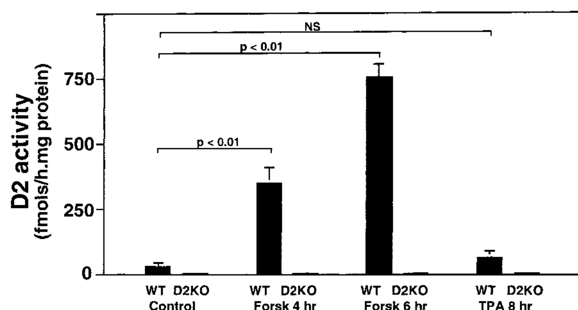

Fig. 4. D2 Activity in Cultures of Astrocytes Obtained from 2-d-Old WT and D2KO Mice

Before harvesting, astrocytes were treated with 10 μM forskolin for 4 h and 6 h, 0.1 μM TPA for 8 h, or vehicle. D2 activity was determined, using 1 nM T_4 as substrate, in sonicates of the astrocytes prepared as described in *Materials and Methods. Bars* indicate the mean $\pm$ SE of values obtained in three cultures.

Levels of T_4, T_3, and TSH in Serum from D2KO and WT Mice

Serum T_4 levels, determined in adult mice at 10–12 wk, were approximately 40% higher in D2KO mice than in the WT mice, and this increase was associated with a 2-fold increase in TSH levels (Fig. 7). These differences were highly significant and were observed in both male and female mice. In contrast, serum T_3 levels in D2KO and WT mice were comparable.

To determine whether the elevated serum T_4 levels in the D2KO mice were independent of the rate of T_4 secretion, male D2KO and WT mice were treated with MMI/ClO_4 for 6 wk, a treatment that resulted in undetectable serum T_4 levels and markedly elevated TSH levels. As in the euthyroid mice, levels were higher in the D2KO than in the WT mice (12,799 $\pm$ 1,203 *vs.* 7,126 $\pm$ 567; $P < 0.001$). After 4 wk of MMI/ClO treatment, some of the mice were implanted with a pellet of T_4 designed to release a constant amount of the hormone over a 21-d period. Two dose levels were employed: T_4 (low) yielded a subnormal serum T_4 level; T_4(high) yielded a serum T_4 level that was approximately twice that in untreated euthyroid control mice. The levels of T_4 in serum were determined 14 d after pellet implantation (Fig. 8). As found previously, T_4 levels were significantly higher in the control D2KO mice than in the control WT mice. Serum T_4 levels were also significantly higher in the D2KO than in the WT mice after treatment with MMI/ClO_4 plus exogenous T_4. Since after MMI/ClO_4 treatment, the thyroid was no longer a significant source of T_4, these findings suggest that the clearance of T_4 from plasma is reduced in the D2KO mice.

To determine whether there is a difference in the plasma thyroid hormone binding activity between WT and D2KO serum, a competitive T_3 uptake test was carried out. The percent uptake of $[^{125}I]T_3$ by the anti-T_3 antibody in the presence of WT and D2KO serum was 49.0 $\pm$ 0.635 and 51.6 $\pm$ 0.83 ($P < 0.05$), respectively. These data indicate that plasma thyroid hormone binding activity in WT serum is slightly but significantly higher than that in the D2KO serum. Thus, the elevated plasma level in the D2KO mice cannot be attributed to a change in the physical state of the hormone in plasma.

TSH Suppression Studies

The observation that both T_4 and TSH levels are elevated in serum of D2KO mice suggests that the pituitary/thyroid feedback system is resistant to T_4. Presumably, this is because, in the absence of D2, the pituitary is unable to convert the T_4 to T_3, resulting in lower thyroid hormone receptor occupancy in the D2KO animals. To test this hypothesis, male WT and D2KO mice were placed on MMI/ClO_4 for 4 wk to raise their circulating TSH levels. D1 activity was inhibited by the administration of PTU. On the day that they were killed, the mice were bled from the tail and 1 h

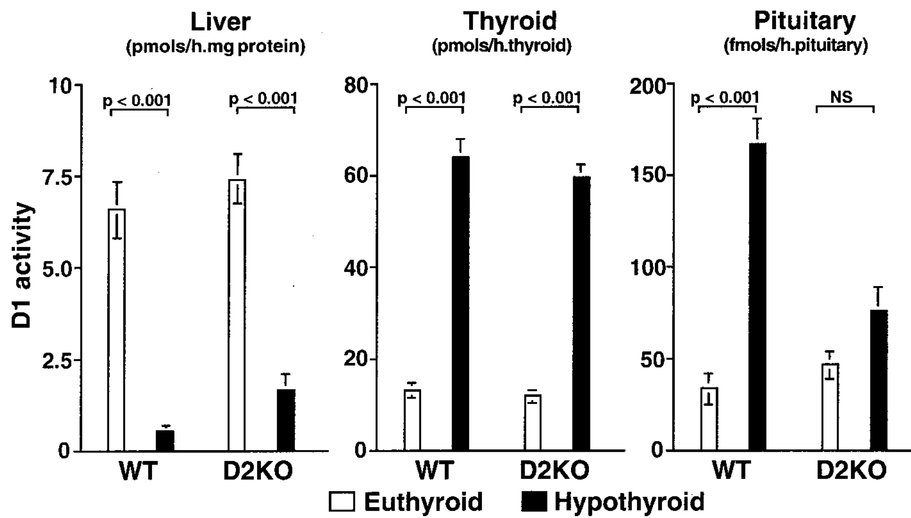

Fig. 5. D1 Activity in Liver, Thyroid, and Pituitary from Euthyroid and Hypothyroid WT and D2KO Mice

Hypothyroidism was achieved as described for Fig. 2. The substrate was 1 nM rT3, and D1 activity was determined as the fraction of the total activity that was inhibited when incubation was carried out in the presence of 1 mM PTU. *Bars* indicate the mean ± SE of values obtained in six (hypothyroid) and eight (euthyroid) mice.

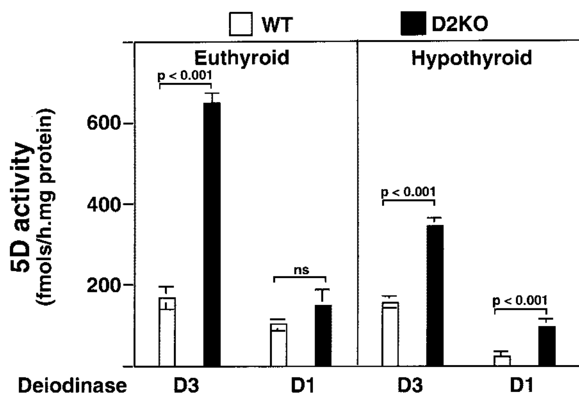

Fig. 6. 5D Activity in Cerebral Cortex of Euthyroid and Hypothyroid WT and D2KO Mice

Hypothyroidism was achieved as described for Fig. 2. The substrate was 1 nM T_3. D3 activity was determined as the fraction of the total activity that was insensitive to the presence of 1 mM PTU. The PTU-sensitive fraction was taken to represent the 5D activity of the D1 enzyme. *Bars* indicate the mean ± SE of values obtained in six (hypothyroid) and eight (euthyroid) mice.

later they were injected sc with T_4 (3 μg/100 g body wt), T_3 (1.2 μg/100 g body wt), or vehicle. They were killed and serum was obtained 5 h later. At the time of death, serum T_4 levels in the WT and D2KO mice injected with T_4 were 7.8 ± 0.99 and 9.9 ± 0.77 μg/100 ml (ns, $P > 0.05$), respectively. Serum T_3 levels in the WT and D2KO mice injected with T_3 were 299 ± 32.4 and 366 ± 32.1 ng/100 ml ($P < 0.05$), respectively. Serum TSH levels in both WT and D2KO mice 5 h after injection of hormone were still at least 10 times those seen in corresponding euthyroid mice. However, values for both the initial and final TSH levels were very variable within each group, and thus data are presented as the final TSH level expressed as a percent of the initial TSH level. Both T_4 and T_3 significantly suppressed circulating TSH levels in the WT mice. In contrast, in the D2KO mice, a significant decrease in circulating TSH was achieved only after the T_3 injection; T_4 had no significant inhibitory effect (Fig. 9).

DISCUSSION

We have described herein the development of a D2-deficient mouse model created by targeted disruption of the *Dio2* gene and have provided unequivocal evidence that D2 activity is absent in tissues from this animal. Indeed, no D2 activity was detected in the tissues of mice carrying two copies of the recombinant allele, even after the mice were subjected to cold exposure or hypothyroidism, conditions that resulted in substantial increases in D2 activity in tissues of WT mice. In addition, D2 activity was increased substantially in WT astrocytes exposed to forskolin, whereas astrocytes from the D2KO mice were devoid of D2 activity either in the absence or presence of this compound.

It has been suggested that the mammalian *Dio*2 gene does not code for a functional protein, and therefore the D2 selenodeiodinase is not responsible for the type 2 deiodinase activity found in rodent tissues (22, 23). This suggestion was based on indirect evidence whereby investigators using an antibody raised against the carboxy terminus of the rat D2 selenodeiodinase were unable to detect a protein of the predicted 30-kDa size in $(Bu)_2cAMP$-stimulated astrocytes, nor was the antibody able to immunoprecipitate D2 activ-

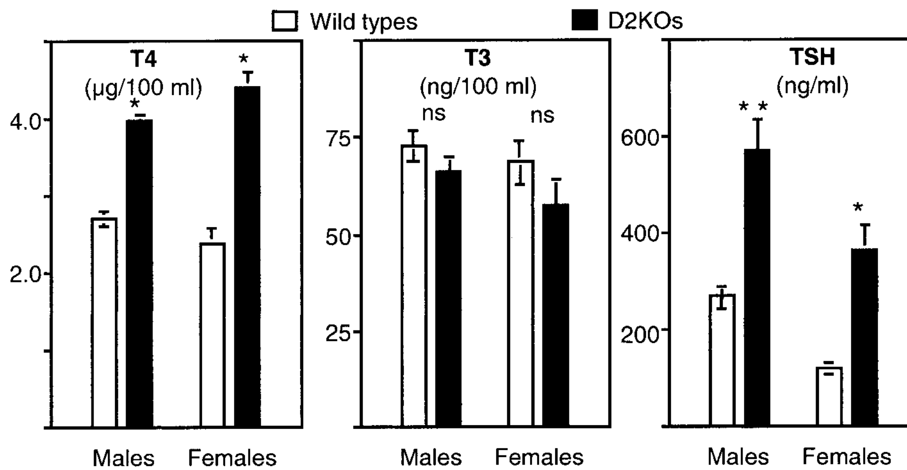

Fig. 7. Levels of T_4, T_3, and TSH in serum of Male And Female WT and D2KO Mice, 10–12 wk Old

Bars indicate mean ± SE of values obtained in a minimum of nine mice. *, $P < 0.001$; **, $P < 0.005$ compared with WT mice; ns, not significant. $P > 0.05$.

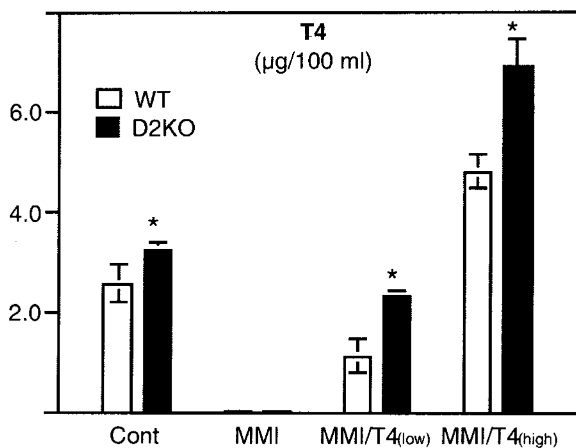

Fig. 8. Levels of T_4 in Serum of Male WT and D2KO Mice 11 wk Old

Cont, Vehicle-treated; MMI: mice made hypothyroid with MMI/ClO_4; MMI/T_4(low) and MMI/T_4(high): mice treated with MMI/ClO_4 and implanted with a T_4 pellet containing, respectively, 0.0025 mg and 0.005 mg T_4. *Bars* indicate the mean ± SE of values obtained in 10 mice. *, $P < 0.05$.

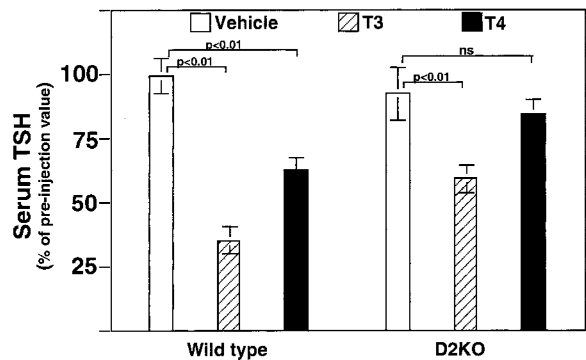

Fig. 9. Suppression of Serum TSH Levels by T_4 and T_3 in Hypothyroid Male WT and D2KO Mice, 10–11 wk Old

Mice were rendered hypothyroid by treatment with MMI/ClO_4 for 4 wk to raise their TSH level. D1 activity was inhibited by administration of PTU in the drinking water for the 5 d before mice were killed. On the day mice were killed, an initial serum sample was obtained from the tail, and then the mice were injected sc with PTU (1 mg/100 g body wt). One h later they were injected sc with T_4 (3 μg/100 g body wt), T_3 (1.2 μg/100 g body wt), or vehicle. After a further 5 h the mice were killed and a second serum sample was obtained. The serum TSH concentration in the second serum sample is expressed as a percentage of that in the initial sample. *Bars* indicate the mean ± SE of values obtained in 10 or 11 mice.

ity out of sonicates derived from these same cells. However, the results presented here provide definitive evidence that the product of the *Dio2* locus is essential for the expression of D2 activity in mammalian tissues. Given that the expressed protein product of this gene has been demonstrated to catalyze deiodination with properties identical to that of the D2 activity found in native tissues (24), there can be little doubt that the D2 selenodeiodinase is responsible for this enzyme activity *in vivo*. The inability of antibodies to detect this protein in cell sonicates likely relates to its known very low abundance.

The most striking features of the D2KO mouse are that, compared with the WT mouse, the circulating levels of both T_4 and TSH are elevated while those of T_3 are unchanged. The fact that the levels of TSH and T_4 are both raised in this model suggests that the pituitary thyrotrophs are not responding normally to the plasma level of T_4. The most likely explanation for this is that, in the absence of D2 activity, the ability of the pituitary to convert T_4 to T_3 is impaired. This results in a decrease in the intrapituitary T_3 level, and hence nuclear TR occupancy is reduced, which leads to a decreased inhibition of TSH synthesis and secretion.

Direct evidence that the pituitary of the D2KO mouse is resistant to T_4 is provided by the finding that

plasma TSH levels in hypothyroid WT mice were suppressed significantly after injection of either T_4 or T_3, whereas in hypothyroid D2KO mice significant suppression of plasma TSH occurred after injection of T_3 but not after injection of T_4. This lack of suppression by T_4 in the D2KO mouse occurred even though a higher serum T_4 level was attained in the D2KO mice than in the WT animals. These data provide unequivocal evidence that pituitary D2 activity is essential for the participation of circulating T_4 in the pituitary/thyroid feedback mechanism.

The degree of impairment of the pituitary/thyroid feedback mechanism in the euthyroid D2KO mouse is relatively modest since much higher plasma TSH levels were obtained when the mice were rendered hypothyroid. This suggests that the system is only partly dependent on the presence of D2 activity. This was expected since it has been shown previously that only 55% of the T_3 bound to the pituitary TRs is generated by 5′D within the pituitary (25). An additional complicating factor is that the euthyroid rat pituitary gland expresses D1 as well as D2 (26–29). In the present study, PTU-sensitive D1 activity was demonstrated in both WT and D2KO mice using rT_3 substrate, but not when 1 nM T_4 was substrate. This latter finding supports the concept that, in these mouse tissues, D1 contributes only in a limited fashion to intrapituitary T_3. However, one must be cautious about extrapolating to the *in vivo* situation data obtained in *in vitro* assays using cofactors and conditions that presumably are not the ones employed *in vivo*.

There are several possible explanations for the elevated plasma T_4 levels in the D2KO mouse. Under normal conditions the steady-state plasma T_4 level is determined by the rate of thyroidal secretion of T_4, the binding activity of the plasma T_4 binding proteins, and the rate of T_4 clearance from the circulation by peripheral tissues. The clearance of T_4 from plasma is dependent on multiple factors including the activities of the three deiodinases, and of the sulfatases and glucuronidases that catalyze the formation of T_4 conjugates, some of which are cleared in bile and feces. The elevated plasma T_4 level in the D2KO mouse cannot be attributed to an increase in plasma T_4 binding activity since binding activity was shown to be slightly lower in the D2KO compared with the WT mouse. Since the plasma TSH level is elevated in the D2KO mouse, thyroidal secretion of T_4 is likely to be increased. However, the plasma T_4 level was also elevated in the hypothyroid D2KO mice implanted with a constant release T_4 pellet. In these mice, secretion of endogenous T_4 had been reduced to levels that were undetectable. While this elevated plasma T_4 level could be due to a reduction in the volume of distribution of T_4 in the D2KO mouse, this seems unlikely since the WTs and D2KO mice were of comparable weight, and their plasma T_4 binding activities were similar. A more likely explanation is that the clearance of T_4 from plasma of the D2KO mice was decreased. This reduced rate of clearance cannot be attributed to reduced D1 activity because, under all conditions studied, D1 activity in D2KO mice and corresponding WT mice was comparable. Thus, the reduced clearance of T_4 from plasma under the conditions of this experiment likely results, at least in part, from the absence of D2 in peripheral tissues. The reduced clearance of T_4 occurred in spite of the increase in brain D3 activity, a change that would be expected to increase the clearance of plasma T_4.

Despite the absence of D2 activity, serum T_3 was maintained close to WT levels in D2KO mice. One explanation for this finding is that the T_3 generated from T_4 by D2 activity does not normally contribute to the plasma T_3 level. If true, the data would support the view that the role of the D2 is to generate T_3 from T_4 primarily for use within the cell or tissue in which the deiodination has occurred (30, 31). However, there is considerable indirect evidence consistent with the view that D2 does in fact contribute to plasma T_3. Thus, in rats treated with PTU, which greatly reduced hepatic and renal D1 activity, extrathyroidal conversion of T_4 to T_3 was reduced by only 60–70% (32, 33). Furthermore, the D2 pathway appears to be the predominant mechanism for extrathyroidal production of T_3 in the euthyroid neonate and the only demonstrable pathway in the hypothyroid neonate (34). In addition, a recent study has provided quantitative evidence that a significant fraction of the plasma T_3 in rats is derived from T_4 by the action of D2 (15). If this is the case also in mice, then it must be that the deficient production of T_3 resulting from the absence of D2 activity is compensated for from other sources. Possible sources include T_3 secreted by the thyroid gland *per se* and T_3 generated from T_4 by D1 activity. In the D2KO mouse the conditions are such that the supply from both sources could be enhanced. With the elevated plasma TSH levels, thyroidal secretion of T_3 would be expected to be elevated. In addition, although the levels of D1 activity in liver and thyroid were not altered in the D2KO mouse, T_4 levels are increased, thus providing additional substrate for the generation of T_3. Another factor that could contribute to the maintenance of plasma T_3 levels in the D2KO mouse is a possible reduction in the rate of clearance of T_3 from plasma. Studies are in progress to resolve these issues.

D3 activity was increased approximately 3-fold in cerebral cortex of D2KO mice, a paradoxical increase that would seemingly further compromise T_3 levels in the CNS. The stimulus for this increase is not clear. It may result from differences in intracellular levels of T_4 and T_3 in the CNS. It has been shown that the amphibian D3 gene is up-regulated directly by thyroid hormone (4, 35) and D3 activity in rat brain also appears to be stimulated by thyroid hormone (36). However, the increase in plasma T_4 in the D2KO mouse would not be expected to result in an increased level of T_3 in the brain, since this organ is thought to derive the majority of its T_3 from plasma T_4 by local D2 activity (25). One possible explanation for the increase in D3 activity is that T_4 *per se* has a stimulatory effect on the expression of the D3 gene, or an inhibitory effect on the degradation of the enzyme or its mRNA.

It is also possible that the set point of D3 activity is altered in the D2KO mice during development. D3 activity in rodent brain decreases markedly in the neonatal period (37), and the extent of this decrease may be altered in mice deficient in D2. Further studies, including those in which intracellular T_4 and T_3 levels in different parts of the brain are determined, are in progress to resolve this question.

Aside from the increased plasma T_4 and TSH levels and the pituitary resistance to T_4, the D2KO mouse exhibits a seemingly mild phenotype. Reproductive capacity is unimpaired, any abnormalities in growth are both small and transient, and, outwardly, the mice appear as mobile and healthy as the WT mice. This suggests that the thyroid hormone-dependent processes that participate in these functions are able to derive sufficient T_3 from the plasma, a source that is not compromised in the D2KO mouse. However, it is possible that other crucial TH-dependent functions such as hearing, vision, learning, and memory, functions that are less obvious on gross inspection, may be more sensitive than locomotive function to a decrease in the T_3 level in brain and may, in fact, be impaired in the D2KO mouse. These parameters are currently being studied.

The D2KO mouse was able to survive in the cold. This is notable since the ability of normal rodents to survive sudden and/or prolonged exposure to cold is thought to be due primarily to increased heat production in BAT (38), a response that does not occur in the absence of T_3 (14). In fact, hypothyroid rodents cannot survive in the cold (39). D2 is the only deiodinase expressed in BAT, and BAT D2 activity is up-regulated rapidly and substantially when rodents are placed at 4 C, to enhance the local production of T_3 from circulating T_4 (40). Thus, the finding that D2 expression in BAT is not essential for survival in the cold indicates that either BAT is not completely dependent on local generation of T_3 from T_4 or that the D2KO mouse can compensate in some way for impaired thermogenic function in BAT.

In summary, targeted inactivation of the *Dio2* selenodeiodinase gene results in the complete loss of D2 activity in all tissues examined. The increased serum levels of T_4 and TSH observed in D2KO mice, together with the finding that T_4 is ineffective in reducing the plasma TSH level in D2KO mice, demonstrate that the D2 is of critical importance in the pituitary/thyroid feedback regulation of TSH secretion. Studies are in progress to determine the extent to which other thyroid hormone-dependent processes, particularly those in the CNS, are impaired in the D2KO mouse.

MATERIALS AND METHODS

Generation of a Mouse Lacking D2 Activity

A 129/SvJ mouse genomic library (in Lambda DASH II; Stratagene, La Jolla, CA) was probed with the entire coding region of the rat D2 cDNA (24). A 13.5-kb *Dio2* genomic fragment, designated 8B, was isolated and subcloned into pBluescript SK(−) (Stratagene) for restriction enzyme mapping and partial sequence analysis. Intron/exon junctions were determined from the known sequence of the mouse D2 cDNA (P. R. Larsen, personal communication), and a single, large intron (~8.5 kb) was found, which interrupted the coding region. Additional *Dio2* genomic sequence was obtained from a mouse P1 genomic clone isolated by Incyte Genomics (St. Louis, MO), using a PCR-based screening method with primers based on the 3′-end of the original 8B sequence.

The *Dio2* targeting vector was constructed in pBluescript SK(−) plasmid, in which a 1.7-kb neomycin resistance (*Neo*) cassette had been ligated into the *Eco*RI/*Hin*dIII restriction enzyme site in the sense orientation (provided by Dr. Nancy Speck, Dartmouth Medical School). The cassette contained the *Neo* coding region flanked by the promoter and 3′-untranslated region of the phosphoglycerokinase gene. As diagrammed in Fig. 10, a 5.7-kb, *Bgl*II/*Spe*I restriction fragment of the *Dio2* intron was ligated to the 5′-end of the *Neo* cassette, and a 5.0-kb *Stu*I fragment located 3′ to the D2 coding region was ligated to the 3′-end. The thymidine kinase gene (not shown) was ligated 3′ to the latter, and in the same orientation. The targeting vector did not contain the ~2.6 kb *Spe*I to *Stu*I fragment of the *Dio2* gene, and hence 72% of the D2 coding region was deleted, including the TGA codon, which codes for selenocysteine.

The targeting vector, linearized by *Not*I, was electroporated into the AB2.2 line of 129/SvJ embryonic stem (ES) cells, which were grown in the presence of G-418 and gancyclovir. To screen ES cells for homologous recombination of the disrupted D2 allele, antibiotic-selected ES clones were regrown in duplicate 96-well plates. Genomic DNA was prepared directly in the wells of one plate (41), and the second plate was frozen for future use. Pools of DNA from six wells were then subjected to PCR using the Expand Long Template System (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. The sense and antisense primers were based on the native *Dio2* gene sequence just 5′ of the recombination site, and sequence in the *Neo* cassette, respectively (Fig. 10). This reaction was designed to generate a 6-kb PCR product only if the template contained DNA in which homologous recombination had occurred. As a positive control for generating such a 6-kb product, PCR was carried out using wild-type ES cell DNA, the same sense primer, and an antisense primer based on sequence in an equivalent position of the native gene. The PCR products were separated by electrophoresis and when a 6-kb band was detected, the six samples of DNA in that pool were subjected individually to the same PCR procedure to determine in which clone(s) homologous recombination had occurred.

Positive PCR results were confirmed by Southern analysis. The ES cell genomic DNA samples were digested with *Stu*I, electrophoresed on 0.8% agarose, and transferred to Nytran (Schleicher & Schuell, Inc., Keene, NH.). Blots were probed with the 1.2-kb *Stu*I/*Bgl*II fragment that is located in the *Dio2* gene just 5′ of the recombination site (Fig. 10). Because there is a *Stu*I site in the *Neo* cassette, the D2 knockout and wild-type alleles yield bands of 7 and 9.3 kb, respectively.

Two targeted ES clones were obtained and injected into blastocysts, which were in turn implanted into CD-1 pseudopregnant mice. The resulting chimeric male pups were crossed with C57BL/6 females for determination of germ line transmission of the mutated allele in the F1 generation. The genotyping was accomplished by Southern analysis (see above) of genomic DNA obtained from tail tips and prepared using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). The founder males were then bred to the +/− F_1 females, and the −/− (D2KO) and +/+ (WT) genotypes identified in the offspring. Mice of the same genotype were then bred to generate and maintain colonies of WT and D2KO mice.

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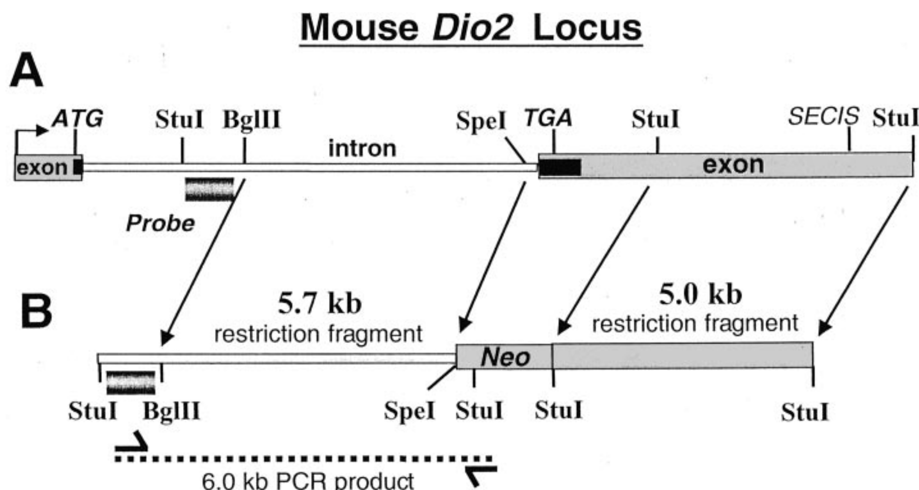

Fig. 10. Targeting Strategy for the *Dio2* Knock-Out Mouse

An approximately 17.3-kb restriction fragment of the mouse *Dio2* gene locus is shown in panel A. The 798-bp coding region is indicated by the *black bar* and contains the TGA codon, which codes for selenocysteine. The selenocysteine insertion sequence (SECIS) is located near the 3′-end of the last exon. The *long arrows* extending to the targeting vector (B) indicate the restriction fragments of the gene that were ligated adjacent to the neomycin cassette (*Neo*). The approximately 3-kb thymidine kinase gene (not shown) was inserted just 3′ of the downstream *Stu*I site of the 5.0-kb arm. A *Not*I site in the multiple cloning region of the phagemid was used for linearization of the targeting construct before its electroporation into ES cells. The oligonucleotide primers and resultant 6-kb PCR product, the presence of which was indicative of homologous recombination in the screening of ES cells, are represented by the *half arrows* and *dotted line*. Confirmation of homologous recombination was achieved by digestion of genomic DNA from the positive ES clones with *Stu*I followed by blot hybridization with the approximately 1.2-kb *Stu*I to *Bgl*II fragment, located just 5′ of the recombination site in panel B. A 7-kb band, which represents the distance from the 5′-*Stu*I site located in the native gene to the *Stu*I site within the Neo cassette, was expected on the autoradiograph in combination with the WT fragment. The latter is a 9.3-kb fragment that is generated by cleavage at the first two native *Stu*I sites shown in panel A.

Animals

In addition to the mice described above, 10-wk-old male and female mice of each of the two background strains, 129SV and C57Bl/6, were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under conditions of controlled lighting and temperature in the barrier section of the Dartmouth Medical School animal research facility. Details of all births, including litter size, birth abnormalities, and neonatal deaths were recorded. Mice were weighed at regular intervals. Some mice were exposed to 4 C for 4 or 16 h before study. Others were made hypothyroid by placing them on drinking water containing 0.1% MMI and 1% $KClO_4$ (MMI/ClO_4) for a minimum of 4 wk before study. Some of the latter mice were also implanted sc with a pellet containing either 0.0025 or 0.005 mg T_4. These pellets were designed to yield a constant level of plasma T_4 over a 21-d period and were custom-made for us by Innovative Research of America (Sarasota, FL). Preliminary studies indicated that a 0.0025 mg pellet yielded a subnormal plasma T_4 level, whereas a pellet containing 0.005 mg T_4 yielded a plasma T_4 level that was approximately twice the value observed in a WT euthyroid mouse.

In another study, male WT and D2KO mice were placed on MMI/ClO_4 for 5 wk. Five days before the mice were killed, PTU was added to their drinking water (1 mg/liter). On the day of death, blood (~150 μl) was obtained from the tail after which they were injected sc with PTU (1 mg/100 g body wt). The PTU was administered in 0.1 ml of saline containing 0.01 N NaOH. One hour later they were injected sc with T_3 (1.2 μg/100 g body wt), T_4 (3 μg/100 g body wt), or vehicle. The hormones were administered in 0.1 ml of saline containing 0.1% BSA (wt/vol). The animals were killed 5 h later.

Except when stated otherwise, all mice were between 9 and 12 wk of age at the time they were killed for study. All animal protocols were approved by the Institutional Review Board of Dartmouth Medical School.

Tissue Preparation

The mice were killed by ether anesthesia followed by exsanguination. Once the mice were anesthetized, the abdomen was opened and blood was taken directly into a syringe from the inferior vena cava. The serum was obtained by centrifugation and then stored at −20 C for subsequent assays. The following tissues were obtained: liver, kidney, BAT, thyroid (plus the section of trachea to which it was attached), cerebrum or cerebral cortex, and pituitary. Liver, kidney, BAT, and brain tissue were homogenized in 0.25 mM sucrose; 20 mM Tris-HCl, pH 7.6, containing 5 mM dithiothreitol (DTT) as previously described (42) to yield approximately a 1:5 homogenate (wt/vol). Pituitary and thyroid (plus trachea) were homogenized by hand in 0.5 ml of the same buffer using a ground-glass homogenizer. The homogenates were centrifuged at 1,000 × *g* for 15 min and the supernatants stored at −20 C for subsequent assay of 5′D and 5D activities.

In addition, total RNA was isolated from BAT obtained from D2KO and WT mice after exposure to 4 C for 16 h, using a commercial RNA isolation reagent (TRIzol Reagent, Life Technologies, Inc., Gaithersburg, MD), according to the manufacturer's instructions. Poly(A)$^+$RNA was isolated from total RNA as previously described (43).

Preparation and Culture of Astrocytes

Cerebral hemispheres were removed from 2-d-old WT and D2KO mice and primary cultures of astrocytes were prepared as described previously (44). Briefly, cells were cultured in DMEM supplemented with 6 g/liter glucose, 2.4 g/liter

$NaHCO_3$, antibiotics (100 U/ml penicillin, 100 μg/liter streptomycin, and 0.25 μg/liter Amphotericin; Sigma, St. Louis, MO) and 10% FCS (DMEM/FCS). The medium was changed every 2–3 d until cells reached confluency at approximately 10 d. At this stage, the DMEM/FCS was removed and cells were washed with a 1:1 mixture of DMEM and Ham's F12 (DMEM/F12), supplemented with 5.2 g/liter glucose, 1.8 g/liter $NaHCO_3$, and the antibiotics as listed above. The cells were cultured for 3 additional days in DMEM/F12 supplemented with 30 nM sodium selenite, 10 μg/ml insulin, 10 μg/ml transferrin, followed by 1 additional day in DMEM/F12 supplemented with 30 nM sodium selenite, 1 μM cortisol, and 10 μg/ml transferrin. Some cultures of astrocytes were treated with 10 μM forskolin for 4 h and 6 h, or 0.1 μM tetradecanoyl-phorbol-13-acetate (TPA) for 8 h before harvesting.

At the time of harvesting, the medium was aspirated, the cells were rinsed twice with 3 ml of ice-cold PBS, and the dishes containing the cells were frozen at −80 C. Cells were processed for assay of D2 activity by scraping the cells into 1 ml of Tris/sucrose buffer. Cells were centrifuged at 500 × *g* for 3 min and then resuspended in 50 μl of Tris/sucrose buffer and sonicated for 5 sec.

Determination of 5′D and 5D Activities

5′D and 5D activities were assayed according to our published methods (45, 46). Briefly, for 5′D activity the reaction mixture (total volume 50 μl) contained between 2 and 100 μg tissue protein and 1.2 mM EDTA. The substrate was 1.0 nM of either $[^{125}I]rT_3$ or $[^{125}I]T_4$, and the cofactor was 20 mM DTT. Incubations were carried out for 1 h at either 37 or 0 C. The percent deiodination of substrate that occurred at 37 C was corrected for any nonenzymic deiodination by subtracting that which took place during the same time period at 0 C. For liver, kidney, BAT, cerebrum, and astrocytes, 5′D activity is expressed as picomoles or femtomoles iodide generated/h·mg protein. For pituitary and thyroid, it is expressed per individual thyroid or pituitary gland. In determining 5′D activity, the percent iodide generated was multiplied by 2 since the specific activities of the labeled products were only half that of the substrate. In pituitary and cerebrum, tissues that express both D1 and D2, the 5′D assays were carried out in the presence and absence of 1 mM PTU; at this concentration, PTU inhibits the activity of D1 but not that of D2. Pituitary and cerebrum were also assayed for 5′D activity using $[^{125}I]T_4$ as substrate. T_4 is the preferred substrate for D2 and, at the 1.0 nM concentration employed in the assay, none of the 5′D activity was PTU sensitive, indicating that it was all attributable to D2.

For determination of 5D activity, the reaction mixture (50 μl) did not contain EDTA, the substrate was 1.0 nM $[^{125}I]T_3$, and the cofactor was 50 mM DTT. Assays were conducted in the presence and absence of 1 mM PTU. In both the 5D and the 5′D assays, protein concentrations were adjusted to ensure that deiodination was less than 20%.

$[^{125}I]rT_3$, $[^{125}I]T_4$, and $[^{125}I]T_3$ (specific activities ~1,000 μCi/μg) were obtained from Perkin-Elmer Corp. (Norwalk, CT) and were purified by chromatography using Sephadex LH-20 (Sigma) before use. Protein concentrations of all samples were determined according to the method of Comings and Tack (47) using BSA as the standard.

Assays for Serum T_4, T_3, and TSH

Serum total T_4 and T_3 levels were determined using the Coat-A-Count RIA total T_4 and total T_3 (Diagnostics Systems Laboratories, Inc., Webster, TX). The total T_4 assay was carried out according to the manufacturer's instructions. Tests with serum obtained from thyroidectomized mice indicated that there was no nonspecific effect of mouse serum in this T_4 assay. The minimal detectable concentration of T_4 in the assay was 0.25 μg/100 ml. The total T_3 assay required modification for use with mouse serum. Test studies with charcoal-stripped mouse serum (48), unsupplemented and supplemented with 50 or 100 ng T_3/100 ml, revealed a significant nonspecific effect of mouse serum in the assay; the maximum binding value ($[^{125}I]T_3$/antibody binding in the absence of nonradioactive T_3) was suppressed approximately 12%. This resulted in spuriously high T_3 levels in the supplemented serum samples. Correction of the maximum binding value for this nonspecific effect resulted in T_3 levels in the supplemented samples that were within 5% of the estimated levels. This correction was applied to all mouse serum samples. The minimal detectable level for T_3 was 7 ng/100 ml. The plasma thyroid hormone binding activities in WT and D2KO sera were compared using the Coat-A-Count T_3 uptake kit purchased from the same company. In this assay, the samples of sera were incubated with $[^{125}I]T_3$ in tubes in which a T_3-specific antibody had been immobilized to the wall. The percent uptake of the $[^{125}I]T_3$ by the antibody is determined.

Mouse serum TSH levels were determined using a highly sensitive double antibody method, developed by A. F. Parlow. The assay used a highly purified rat TSH (AFP11542B) as the iodinated ligand, a selected guinea pig antimouse TSH (AFP98991), at a final tube dilution of 1:500,000, as the primary antibody, and a partially purified extract of mouse pituitary containing TSH (AFP5171.8MP) as the reference preparation. Cross-reactivity of either highly purified mouse FSH or mouse LH in this mouse TSH RIA was less than 1%. Displacement curves obtained by testing sera of hypothyroid mice in graded dilutions did not depart significantly from parallelism with displacement curves for the reference preparation. Recovery of exogenous mouse TSH activity added to mouse serum was 80–100%. The coefficients of variation within and between assays were 5% and 11%, respectively. Serum of euthyroid WT and D2KO mice were generally tested using a volume of 40–80 μl per RIA tube, whereas sera of hypothyroid mice could be tested in volumes as small as 5 μl per RIA tube. All mouse sera were assayed for TSH activity in the absence of knowledge by the tester of the treatment status of the donor mice. Values for serum TSH levels in euthyroid mice obtained using this method, and the finding that levels are significantly higher in male than in female mice, have been reported previously (49).

Northern Analysis

Northern analysis was carried out as described previously (43). Between 5–6 μg of $Poly(A)^+$RNA were loaded into each lane. The blot was probed with a 950-bp mouse D2 cDNA that contained the entire coding region plus some 5′-untranslated region (17). To document the relative amounts of RNA in each lane, the blot was also probed with a cDNA for the unregulated gene, cyclophilin. Hybridizations were carried out at 42 C and the final washes were performed at 60 C.

Statistical Analyses

Data are expressed as mean ± SE. Statistical analyses were carried out using the GB-Stat PPC 6.5.4 computer program (Dynamic Microsystems, Inc., Silver Spring, MD). For comparison of values obtained in D2KO and WT mice, the *t* test was used. For multigroup comparisons, one-way ANOVA was performed, and the differences were assessed using Fisher's least significance difference test. Statistical significance is defined as $P < 0.05$.

Acknowledgments

The authors gratefully acknowledge Ms. Jennifer Fields and the Dartmouth Transgenic Mouse Facility for assistance in producing the D2 mutant mice, and Ms. Rosalie Belcher for

developing and maintaining the mouse colonies and for her excellent technical assistance throughout the study.

Received May 18, 2001. Accepted August 16, 2001.

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This work was supported by USPHS Grants HD-09020 (to V.A.G.) and DK-42271 (to D.L.S.).

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